Studies with Plasmids 1964-1982

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Thesis submitted for the Degree of Doctor of Science in the University of Edinburgh, 1982.

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Abstract

This thesis is concerned with the use of plasmids as genetic tools, and with their structure, replication, transfer and molecular relationships. The two principal plasmids studied have been the sex factor F from Escherichia coli and the TOL plasmid pWW0 from Pseudomonas putida. The first group of papers is concerned with the recombination events involved in Hfr and F' formation; in particular the importance of homologous (Rec-dependent) recombination involving insertion sequences was demonstrated.

Two families of Hfr strains had a key role in the demonstration that E.coli replication is bidirectional. Other papers concerned with replication showed that DNA polymerase III is involved in F replication; that most cases of co-habitation of incompatible plasmids are due to recombination; and considered models for the segregation of incompatible plasmids during cell growth.

Time of entry experiments for transfer of the whole chromosome were used to establish that the basic co-ordinates of the published E.coli map were reasonable. A series of papers analysed the efficiency and complexity of Hfr x F^- matings and of plasmid transfer through recipient populations. This work demonstrated the intrinsic high efficiency of cell-cell interactions involved in mating, but also the constraints on further matings within mixed bacterial colonies in Hfr matings and from cells that have received plasmids, even if these plasmids are genetically de-repressed.

Studies on the degradative plasmids have aimed at a detailed understanding of a particular TOL plasmid, pWW0. This has involved development of reliable isolation procedures, construction of restriction endonuclease maps, and the cloning of fragments. A variety of structural changes of pWW0 have been studied: these include the loss of the Tol^+ function by a specific

excision event, interactions between pWW0 and the chromosome, and the formation of hybrids between it and R plasmids.

Restriction endonuclease digestion and DNA hybridisation have been applied to the investigation of relatedness of independently isolated plasmids. It has been shown that different TOL plasmids can be very closely related, and also that R plasmids and degradative plasmids belonging to the same incompatibility group have related sequences. Such data, and those from other laboratories, have been drawn together in a book (Section VII) that documents the idea that plasmids, with transposons, provide the variation and new combinations upon which naturalselection acts to give the rapid evolution of bacterial populations, which has major medical and environmental implications.

Introduction

This thesis includes those of my experimental papers published between 1964 and 1982 that use plasmids as genetic tools, or investigate aspects of their replication, structure, transfer and molecular relationships. The initial papers concern the sex factor of E.coli, but some of the later ones are concerned with a wider range of plasmids and more molecular methods of analysis. This reflects the pattern of research in molecular genetics, to which plasmids have contributed so much; it was originally focussed primarily on E.coli and a few other organisms, but now more systems are being studied, and the role of biochemical methods has become more important. In parallel it has also become evident that plasmids have great importance in the natural world, in particular in providing variation within bacterial populations upon which natural selection can act. These themes are developed and documented more fully in my book "Plasmids" (Section VII), of which the table of contents is summarised. However, purely review papers and also publications arising from my work on a different topic between 1965 and 1968 are excluded from this thesis.

Summary with Titles and Authors of Publications

Section I. Physical Interactions of F and E.coli chromosome

(1) P. Broda, J.R. Beckwith and J. Scaife. The characterisation of a new type of F-prime factor in Escherichia coli K12. Genetical Research, 5, 489-494 (1964).

In the early 1960's two examples were known in E.coli of reversible integration of "episomes" into the chromosome. One was phage λ, where the process of integration is the basis of lysogenisation, and aberrant excision results in the formation of λ transducing particules. The other was F, where integration yields Hfr clones, and aberrant excision yields F' plasmids. Both have had great importance in molecular genetics, for instance because they are the two classical systems for complementation in E.coli. The mechanism of integration itself revealed, with λ, site specific recombination and, with F, insertion (IS) sequences. The latter will be referred to in later papers discussed in this section.

The F' factor was isolated in Lederberg's laboratory: other F' factors were selected for by transfer from the Hfr strain to other strains; F13 was unusual in being demonstrated in the strain in which it had arisen, W3747. This paper describes the analysis of F13 (a) in strain W3747 and (b) when transferred to other strains. Its properties differed in the two cases as if W3747 was still haploid for the F13-borne markers, lac, tsx and ade; growth of W3747 with acridine orange, which normally (and in other F13 carrying strains) causes curing here results in F13 re-integration at other sites, yielding Hfr strains. We concluded that there was no chromosomal homologue of the DNA of this plasmid, and that therefore it had arisen by a simple reciprocal recombination event in the parental strain. Of the markers carried lac was on the opposite side of the point of origin of the Hfr strain from tsx and ade, so that F13 was the first reported example of an

F' plasmid carrying proximal markers, or indeed both proximal and distal markers. Mapping indicated that these bore the same relations to each other and F as in the parental Hfr. The model of formation we proposed was consistent with the recently advanced Campbell model for excision of λ. It also made likely the view that F is circular and that in Hfr formation it is linearly inserted into the chromosome, neither of which had been evident. This work was the first support for the Campbell model from a system other than λ. Because it carries the whole of F inserted with chromosomal DNA, F13 was later used by Davidson's laboratory in their heteroduplex analysis of Hfr and F' formation (Hu, Ohtsubo and Davidson, J. Bacteriol. 122 749-763, 1975).

(2) P. Broda. The formation of Hfr strains in Escherichia coli K12. Genetical Research, 9, 35-47 (1967).

The questions posed in this paper were whether the F integration events that yielded Hfr clones could occur randomly on the E. coli chromosome, and whether such Hfrs were a sufficient explanation for F^+ fertility. A non-selective method of isolation yielded 12 Hfrs; careful characterisation revealed that there had been repeated isolation of Hfr classes, indicating regions for preferred integration that can now be understood in terms of chromosomal IS sequences. One class (strains B7, B9, and B10) had origins close to trp, now known to be the region where replication terminates, and where Hfrs do not arise easily. Reconstruction experiments suggested that the Hfrs of the types isolated could only explain about 20% of F^+ fertility; this point was discussed in Willetts and Broda (Ciba Foundation Symposium, 1968) and further data relevant to this point are given in ref 5.

(3) P. Broda. Hfr formation and F^+ fertility. Soc. Gen. Microbiol. Quarterly, 7, 23 (1979).

Strains B7, 9 and 10 referred to above were noted in (2) to have slower growth rates, whereas F^+ revertants from them grow normally. This could be due to integration causing a specific lesion. However an alternative, that integration in this general segment of the chromosome is deleterious, is attractive since as noted above Hfrs with origins here are uncommon. This abstract and Table 1 give further evidence on this point. A survey was made of Hfr strains isolated by using F'_{ts} lac$^+$ integration into the chromosome of a strain with a large deletion in the lac-pro region and therefore lacking homology with the plasmid. It was found that only 11% of the Hfr strains isolated had origins between 2' and 44' on the conventional E.coli map, whereas 83% had origins in the comparable 44'-85' segment. However the complication that the distribution of origins differ between the Hfrs with the two orientations of transfer should also be noted.

Table 1. Distribution of origins of 159 strains isolated at three separate times from strain ED903.

Map position		2	12	27	44	61	68	85	2
		leu	purE	trp	his	lys	argG	metE	leu
Map length of interval		10	15	17	17	7	17	17	
		direction of transfer ←							
Isolation 1	(31)	0	0	0	1	20	10	0	
2	(7)	2	0	0	0	3	2	0	
3	(54)	1	1	0	2	30	20	0	
Total isolated	(92)	3	1	0	3	53	32	0	
		direction of transfer →							
Isolation 1	(13)	0	0	1	2	10	0	0	
2	(18)	0	0	5	0	3	0	10	
3	(36)	0	3	3	20	10	0	0	
Total isolated	(67)	0	3	9	22	23	0	10	
% of total Hfr isolates		2	3	6	16	47	20	6	

The strains are classified according to the markers that they transfer with high efficiency in crosses with strains JC411 and X478, and their polarities of transfer. Strain ED903 carries a lac pro deletion and F_{ts} lac plasmid. Separate single colonies grown at 30°C were streaked on EMB lactose plates which were then incubated at 42°C. Stably Lac$^+$ clones, each from a separate "founder" lac pro/F_{ts} lac$^+$ colony, were characterised in patch matings and tube matings. In addition, interrupted matings were used with representative strains. Three separate series of isolations were undertaken.

The resulting strains had the designations ED1001-1053, ED2421-2443 and ED3001-3120. The parental strain was initially spectinomycin-resistant but the subcultures used to isolate the second and third series were spectinomycin-sensitive revertants, but were apparently similar in other respects. Map positions of the markers are taken from the 100' map of B. Bachmann and K.B. Low, Microbiological Reviews 44, 1-56, 1980.

(4) P. Broda and P. Meacock. Isolation and characterisation of Hfr strains from a recombination-deficient strain of Escherichia coli. Molec. General Genetics, 133, 166-173 (1971).

It was widely believed that Hfr strains are more difficult to isolate in RecA$^-$ strains than in Rec$^+$ strains. Indeed none had been described. The approach using an F_{ts} lac$^+$ plasmid in a Δ lac-pro strain, referred to above, was also employed to select Hfr clones in a recA$^-$ strain. Several Hfr strains were isolated and it was shown that their transfer properties were similar to those of their recA$^+$ analogues. However, the method of isolation did not allow quantitation of the process, and it could be argued that the use of an F' rather than an F plasmid did not represent a proper test of Hfr formation. Nevertheless, it has been recognised that this example of "illegitimate recombination" (doubly, as there was no homology and no recA$^+$ allele) anticipated the demonstration of a role for IS sequences in Hfr formation.

(5) J. Cullum and P. Broda. Chromosome transfer and Hfr formation by F in rec$^+$ and recA strains of Escherichia coli K12. Plasmid, 2, 358-365, (1979).

This question of the role of the Rec function in Hfr formation was examined more critically in this later paper, which also verified the report by Curtiss and Renshaw (Genetics 63, 7-26, 1969) that certain strains of E.coli K12 (which they termed Type II strains) do not yield stable Hfr strains. We sought Hfr strains in Type I (normal) and Type II strains containing the same F factor, and in their recA$^-$ derivatives. It was shown first that the Rec$^+$ F$^+$ strains were equally fertile, and one hundred times

more fertile than their Rec^- analogues. Further, Hfr strains could be isolated from the Type I strains (one hundred times more efficiently in the Rec^+ strain) but not the Type II strains. Thus the data demonstrated that both F^+ fertility and Hfr formation (in the Type I strain) are primarily $RecA^+$ dependent processes. It therefore appeared that the role of IS sequences is generally to provide homology rather than actively to effect recombination. However, the structure of the integrated F DNA in the rare $RecA^-$ strains that were isolated has not been investigated. Evidence was obtained that fertile clones do arise in Type II cultures but are diluted out in growth; that is, a secondary lesion prevents their maintenance as Hfr clones in such F^+ populations.

Section II. Replication of the E. coli chromosome and of F, and the control of F replication

(6) M. Masters and P. Broda. Evidence for the bidirectional replication of the *Escherichia coli* chromosome. Nature New Biol. 232, 137-140 (1971).

Transduction of *E.coli* using phage P1 had been used by various groups to assay the dosages of different genes in donor populations, with the object of establishing the origin of replication. It was generally assumed that replication was unidirectional. We performed similar experiments but interpreted them instead on a bidirectional model. In particular we used a set of F_{ts} *lac*$^+$ strains (see earlier) to demonstrate that the efficiency of transduction of *lac* but not other markers varied with the origin of transfer of the different Hfrs, in the way expected on the bidirectional model. These and other experiments we described showed also that Hfr strains have the same origin of replication as related F^- and F^+ strains, and we proposed an essentially correct origin and terminus for replication. The clarification of the gross features of replication in *E.coli*, including the precise location of the origin, have followed from this paper.

The data on P1 transduction frequencies raised questions on the mechanism of this process (especially the formation of the transducing particles) since the range of frequencies was larger than could be explained on the basis of the effect replication forks have on gene dosage in the donor strains. The mechanism of transduction has been subject of further studies by Dr. M. Masters.

(7) R. Thompson and P. Broda. DNA polymerase III and the replication of F and ColVBtrp in Escherichia coli K-12. Molec. General Genetics, 127, 255-258 (1973).

When polymerase III was shown to be the authentic DNA polymerase for replication in E.coli the question arose whether it also had a role in F replication. Use of temperature-sensitive (ts) mutants of the gene (dnaE) at the non-permissive temperature were likely to give results that were difficult to interpret, in that chromosome replication - as well as plasmid replication - would be affected and it would not be evident whether any effect on plasmid replication was due to a primary or secondary lesion. A genetic approach was therefore adopted, using a strain in which the dnaE allele was mutagenic at the permissive temperature and which also carried a trp$^-$ deletion. An F' plasmid was constructed to carry a revertible trpE mutation. This allowed a comparison of the frequency of Trp$^+$ revertant formation, depending on whether the wild type or dnaE allele was present. It was shown thus, with a minimum of perturbation, that the mutagenic dnaE function acted very efficiently on the plasmid-borne trp gene and therefore that plasmid replication too was due to polymerase III.

(8) W.M. Anthony, R.C. Deonier, H-J. Lee, S. Hu, E. Ohtsubo, N. Davidson & P. Broda. Electron microscope heteroduplex studies of sequence relations among plasmids of Escherichia coli. IX. Note on the deletion mutant of F, FΔ(33-43). J. Molecular Biology, 89, 647-650 (1974).

Work in collaboration with N. Davidson's laboratory using a mutant F

factor I had previously been involved in characterising[a] established that this plasmid carried a substantial deletion. This mutant was of value as a standard molecule in heteroduplex studies, and also, with other deleted plasmids, established by elimination that all essential F replication functions had to be contained within a 10kb region.

(a) Schell, Glover, Stacey, Broda and Symonds. Genet. Res. 5, 483-484, (1963).

(9) F. San Blas, R. Thompson and P. Broda. An Escherichia coli K12 mutant apparently carrying two autonomous F-prime factors. Molec. General Genetics, 130, 152-163 (1974).

Incompatibility is the inability of related plasmids to cohabit; it is regarded as a manifestation of copy number control. In this paper data were reported on attempts to isolate strains that could carry two cohabiting F' plasmids. It was shown that the persistence of apparently diploid strains depends upon an intact Rec system, so that cohabitation presumably depends upon cointegration of the plasmids with each other or with the chromosome. The important features of this paper are the demonstration of this dependence of cohabitation upon the Rec system, an asymmetry in the loss of cohabiting plasmids, and the discussion of the complexity of this problem.

(10) J. Cullum and P. Broda. Rate of segregation due to plasmid incompatibility. Genetical Research 33, 61-79 (1979).

We calculated the rates of segregation due to plasmid incompatibility under several simple models. A common feature of all the models that we considered is that incompatibility is caused by the inability of the segregation mechanism to distinguish between two incompatible plasmids. We then measured the rate of segregation due to incompatibility of a pair of ColEI derivatives under two conditions: (1) One plasmid was introduced into cells carrying the other by conjugation. (2) Cells carrying both

plasmids were maintained by selection and then selection was released.

Interpretation of the results was made more difficult by effects of the plasmid on the host cell's growth rate. These experiments gave results in agreement with the predictions of a random pool model. Published results were also in reasonable agreement with this model.

Section III. Hfr transfer

(11) P. Broda. Modified map positions for lac and the pro markers in Escherichia coli K12. J. Bacteriology, 117, 741-746 (1974) and (12) P. Broda and J.F. Collins. Gross map distance and Hfr transfer times in Escherichia coli K12. J. Bacteriology, 117, 747-752 (1974).

A point emerging from paper (6) was that the origin and terminus of the E.coli chromosome were not 180° apart on the genetic map. It later emerged that our assigment of the origin was somewhat misplaced but an alternative at that time was that the origin and terminus were correctly located and that the two arms replicated were of equal size, but that the overall co-ordinates, the assigment of which depended upon different Hfr crosses done in different laboratories, were inaccurate. These two papers tested this possibility and also the question whether different Hfr strains transfer the chromosome with similar kinetics. The first paper was primarily concerned with the detailed mapping of the origin of two Hfr strains (B4 and B8) that had origins on opposite sides of lac and transferred the chromosome with different polarities. It was shown that lac itself had been misplaced, the positions of three pro genes in this region were clarified, and demonstrated that 3' elapse before any chromosome DNA, as distinct from F DNA, is transferred.

The second paper describes crosses using these Hfr strains, in which times of entry of different markers distributed round the chromosome were obtained. It was found that (i) these two sibling Hfrs transferred the

whole chromosome at rates differing by 7%, and (ii) there was a systematic and pronounced slowing of the rate of transfer with time. Nevertheless (iii) with the exception of the lac region itself the overall dimensions of the E.coli chromosome (on the assumption that transfer time for a given strain was a simple function of physical distance) were correct.

(13) J.F. Collins and P. Broda. Motility, diffusion and cell concentration affect pair formation in Escherichia coli. Nature, 258, 722-723 (1975).

Mating between Hfr and F' cells is one of the best studied and most used systems in molecular genetics, e.g., for the analysis of chromosome organisation. Also the F factor has been submitted to detailed genetical analysis. Moreover mating in E. coli is one of the simplest systems for the study of cell-cell interactions. However, neither the mating process per se or the kinetics of plasmid transfer in recipient populations had been submitted to critical quantitave analysis. Papers 13-18 address these questions.

The initial step of mating is cell-cell collision, resulting in pair formation. Motile cells were found to be much more proficient at mating than non-motile cells. To allow determination of the true rate constant of the process for motile and non-motile cells at different concentrations the actual movement of such cells was determined. It could then be calculated that pair formation once collision had occurred was most efficient at very dilute concentrations and with non-motile cells, as if secondary collisions or movement impaired the process. The process under such conditions was exceedingly efficient. Evidence was obtained that movement of the donor was vectorial, perhaps due to chemotaxis. This was not pursued, but more recently chemotaxis in mating has been demonstrated with strepococci (Dunny, Brown and Clewell, Proc. Nat. Acad. Sci., Wash. 75, 3479-3483) (1978).

(14) P. Broda and J. F. Collins. Role of simple and complex aggregates in Escherichia coli Hfr X F^- matings. Genetical Research, 31, 167-175 (1978).

It was evident that at high cell densities such as are normal in Hfr crosses in the laboratory cells will have multiple opportunities for pair formation. These could result in complex mating aggregates and also complicated linkage patterns due not to the mode of recombination per se but the complexity of the matings. Moreover, it was reported by others that complex mating aggregates do arise. This paper examined these questions; we concluded that simple mating pairs predominate and that even in platings of mating pairs onto nutrient agar further rounds of transfer were unusual. We also presented evidence that the more complex mating aggregates reported by others must have included many artefacts.

Section IV. Plasmid transfer

(15) P. Broda. Transience of the donor state in an Escherichia coli K12 strain carrying a repressed R factor. Molec. General Genetics, 138, 65-69 (1975).

F is unusual among F-like plasmids in being genetically de-repressed for transfer. In examining the kinetics of plasmid transfer it could be asked what role the repression mechanism has in limiting transfer in populations. This was asked in continuing transfer in papers (16) and (17), but this paper is concerned with the mode of initial de-repression. Statistical analysis of yields of progeny from matings with a large number of separate donor cultures (a fluctuation test) revealed that such de-repression was not clonal, but that nevertheless individual cells could transfer to several recipient cells within a cell generation. This

contrast with subsequent transfer (see (16)).

(16) J. Cullum, J. F. Collins and P. Broda. Factors affecting the kinetics of progeny formation with F'lac in Escherichia coli K12. Plasmid, 1, 536-544 (1978) and (17) J. Cullum, J. F. Collins and P. Broda. The spread of plasmids in model populations of Escherichia coli K12. Plasmid, 1, 545-556 (1978).

Spread of plasmids from donor cells through recipient populations is difficult to analyse quantitatively since, as shown above, mating efficiency is concentration dependent. Moreover, it is dependent on the physiological state of the culture (e.g. growth rate); other unknowns were the competence of the donor recipient cells as a function of stage in the cell cycle; the number of times per generation that a donor cell can transfer; and the interval between acquisition by a recipient cell of a plasmid and the point at which it (or its descendants) becomes able to transfer. In the first of these papers tha parameters involved in F'lac transfer from primary donors are discussed. It was shown that donor cells can only transfer during the growth phase, and only once per generation. The establishment of F'lac in some recipient cells could take more than five generations.

The relative contributions of primary and secondary transfer were assessed in the second paper. It was shown that several generations elapse before recipients become proficient donors. It was concluded that the constraints on the process are such that the terms "epidemic" or "wildfire" spread are inappropriate, since after overnight growth it was still possible to have less than 0.1% of the recipient population infected. It was also found that the possession of a repression system, while inhibiting the initial efficiency of transfer from donors, had no

effect on the kinetics of subsequent transfer from secondary donors; that is, although such re-transfer occurs only after several generations the repression mechanism requires even longer to take effect.

(18) J. Cullum and P. Broda. Recipient competence in F'lac matings in Escherichia coli K12. J. Bacteriology, 137, 281-284 (1979).

The above experiments were performed under conditions of limiting donor concentrations; it was assumed that sufficient competent females would be available. A report from another laboratory suggested that not all recipients were competent; this point was examined in our conditions, and it was shown that competence is a function of the female growth cycle, that all recipients have a competent period, and that on average they can mate with two to three donors in a 30' generation.

Section V. pWW0 and other TOL plasmids

The particular interest of the genes Pseudomonas, apart from the clinical importance of P. aeruginosa, is its metabolic diversity. Evidence began to emerge in about 1971 that plasmids contribute to this diversity. By 1974 the TOL plasmid pWW0 of P. putida emerged as a plasmid with experimental advantages, in particular the phenomenon of "benzoate curing" of the Tol^+ function discussed in relation to (20). The work described in this section, together with that from other laboratories, has made TOL the best-understood degradative plasmid. An initial problem was the isolation of plasmid DNA, which had not then been achieved in P. putida.

(19) C. J. Duggleby, S.A. Bayley, M.J.Worsey, P.A. Williams and P. Broda. Molecular sizes and relationships of TOL plasmids in Pseudomonas. J. Bacteriology, 130, 1274-1280 (1977).

Strains with the ability to degrade toluene and the m and p xylenes, where there was genetical evidence that the property was plasmid-borne,

were provided by Dr. P. Williams (Bangor). Plasmids were isolated by a method involving the removal of chromosomal DNA by alkaline denaturation and absorption to nitrocellulose. They were then characterised by electron microscopy and restriction endonuclease digestion (see 26). In some cases the relation of the Tol phenotype with the plasmid was demonstrated by use of transformation; in others the correlation was the loss of both the phenotype and the plasmid on benzoate curing (see 20). It was shown that the TOL plasmids varied in size between 50 and 200Md. Some from independent isolates were clearly related on the basis of size and restriction enzyme cleavage pattern. This was one of the first pieces of evidence that plasmids in strains that are unlikely to have been selected for or disseminated by man (compare with R plasmids) can be closely related, and that therefore there are a finite number of plasmid families.

An interesting aspect (described in Broda et al in "Microbiology 1978", D. Schlessinger, ed., American Society for Microbiology pp 225-6) was that the size of the plasmid in strain MT14 varied in relation to whether or not growth was in selective medium; selection resulted in larger plasmids (mainly 200Md), presumably by a form of "amplification". Subsequent subculture in non-selective medium resulted in the return of the plasmid size to its original value (all 48Md). The general conclusion is that growth in selective medium, with the object of maintaining the plasmid, may lead to the formation of plasmids of sizes that are inconvenient for isolation as intact purified DNA.

(20) S.A. Bayley, C.J. Duggleby, M.J. Worsey, P.A. Williams, K.G. Hardy and P. Broda. Two modes of loss of the Tol function from Pseudomonas putida mt-2. Molec. General Genetics, 154, 203-204 (1977).

Loss of the Tol^+ phenotype can be selected by growth of the strain in benzoate, which can be utilised by either the plasmid specified (meta) pathway or the chromosomal (ortho) pathway; however the latter pathway is not expressed in the presence of a functional meta pathway. With strain PAW1 (carrying the

archetypal TOL plasmid pWW0, which has a size of 78Md) it had been found that cells able to use the ortho pathway (by loss of the plasmid-borne function) overgrew the other cells. In this paper we showed that among a series of benzoate-cured derivatives of strain PAW1 about half had indeed lost the plasmid whereas the others had undergone a specific excision event, resulting in the loss of about 28Md and a novel pattern of restriction fragments.

(21) P. Meulien, R.G. Downing and P. Broda. Excision of the 40 kb segment of the TOL plasmid from Pseudomonas putida mt2 involves direct repeats. Molec. General Genetics 184, 97-101 (1981).

The basis of the specific excision event has been examined using cloned restriction fragments carrying the end points of the region to be excised. Detailed restriction analysis of these regions and electron microscopy of heteroduplexes revealed that the excision event involved a reciprocal recombination event between a pair of 1.4 kilobase segments that were present in direct repeat.

(22) R. Villems, C.J. Duggleby and P. Broda. Restriction endonuclease mapping of DNA using in situ digestion in two-dimensional gels. FEBS Letters 89, 267-270 (1978).

For functional analysis of the pWW0 plasmid an endonuclease restriction map was required. Attempts to secure one were initially hampered by problems of clustering of cleavage sites and of large numbers of fragments with all available enzymes. The method described here was intended to assist in resolving this problem by allowing separation of doubly digested cleavage fragments in two dimensions, with the second cleavage occurring within the agarose gel. Although it was not made use of extensively in our own project the method aroused substantial interest elsewhere.

(23) R.G. Downing, C.J. Duggleby, R. Villems and P. Broda. An endonuclease cleavage map of the plasmid pWW0-8, a derivative of the TOL plasmid of Pseudomonas putida mt-2. Molec. General Genetics, 168, 97-99 (1979)

and (24) R. G. Downing and P. Broda. A cleavage map of the TOL plasmid of Pseudomonas putida mt-2. Molec. General Genetics 177, 189-191 (1979).

The cleavage maps of pWW0-8 (a derivative that had undergone the specific excision referred to in (20) and (21) and of pWW0 itself for the enzymes HindIII and XhoI are presented here. It was noteworthy that target sites that result in the formation of the smaller fragments are clustered in the intact plasmids. These maps, which have been verified by others, are the basis of the extensive molecular analysis of TOL that has proceeded in our laboratory and others in Britain, Germany, Japan and Switzerland.

(25) P. R. Lehrbach, J. M. Ward, P. Meulien and P. Broda. Physical mapping of the TOL plasmids pWW0 and pND2 and various R plasmid-TOL derivatives from Pseudomonas. J. Bacteriol., 152, 1280-1283 (1982).

The isolation of hybrids of R and TOL plasmids led various authors to suggest that the toluene degradation genes are carried on a transposon. We showed here that there was a wide variation in the amount of TOL DNA that different hybrids contained. If transposition is involved, it cannot therefore involve a unique segment of TOL DNA.

(26) P. Meulien and P. Broda. Identification of chromosomally integrated TOL DNA in cured derivatives of Pseudomonas putida strain PAW1. J. Bacteriol., 152, 911-914 (1982).

Some plasmid-free Tol$^-$ strains derived from a TOL-carrying strain have a segment of TOL DNA located chromosomally. Of three independently isolated strains one had one copy of this segment, another had two copies, while a third had none. It was also shown that a minority of cells in this parental strain had a larger amount of TOL DNA chromosomally.

See also additional publication, number 26a, overleaf.

Section VI. Relationships of plasmids

The study of bacterial evolution has special problems, in particular the absence of fossil evidence, so that its course and timescale are not evident.

26a. P.R. Lehrbach, D.J. Jeenes and P. Broda. Characterization by molecular cloning of insertion mutants in TOL catabolic functions. Plasmid 9, in press (1983).

A physical and genetic map of the Tol catabolic region of pWW0 (TOL) was obtained by restriction endonuclease analysis of several DNA insertion mutants (xylA, xylA xylS, xylS, and xylR) of R plasmid-TOL derivatives. In two cases, the inserted DNA was shown from restriction, DNA hybridization, or heteroduplex analysis of cloned Hind III fragments to originate from within pWW0 fragment Hind III-E. The effect of these DNA insertion sequences on Tol catabolic activity and their role in generating structural alterations to the TOL plasmid are discussed.

In addition, the fundamentally different modes of genetic exchange to those of eukaryotes raise the possibility that simple genealogies cannot be constructed, since it is evident that genetic exchange can occur between rather diverse bacteria. The development of molecular genetics has meant that both DNA and proteins from diverse organisms can be compared. Plasmids are a convenient model for studying evolution at the molecular level. The classic studies of Davidson's group demonstrated the power of the heteroduplex method to reveal relationships and the detailed differences between plasmids. However, it is inappropriate for the survey of relationships of large numbers of plasmids.

(27) R. Thompson, S. G. Hughes and P. Broda. Plasmid identification using specific endonucleases. Molec. General Genetics, 133, 141-149 (1974).

The advent of restriction endonucleases that cut DNA at specific sequences prompted this paper, which first suggested their now obvious use (in conjunction with agarose gels) as a test of close relationship of plasmids. It was shown that plasmids known by other criteria to be related yielded common-sized fragments. Using information from Davidson's group of the relationships of F with other plasmids, it was then possible to specify that the extent of divergence before which common bands would no longer be evident was not great. This method is used widely for the screening of plasmid preparations, for instance in epidemiological tests.

(28) A. L. Heinaru, C. J. Duggleby and P. Broda. Molecular relationships of degradative plasmids determined by in situ hybridisation of their endonuclease-generated fragments. Molec. General Genetics, 160, 347-351 (1978).

It was clear from the above that related plasmids might give very few common bands, and for this reason the method was combined with Southern blotting. The application in which this method was demonstrated for plasmids was with degradative plasmids that had no unambiguously common fragment. It was shown that the SAL (salicylate degradation) plasmid had common sequences with both a range of TOL plasmids and also to a minor extent with the OCT

(29) D. Morris and P. Broda. R plasmids R91 and R91a from Pseudomonas aeruginosa share only the gene for carbenicillin resistance. J. Bacteriol. 138, 1036-1037 (1979).

This paper exemplifies the application of the endonuclease and Southern blotting methods to a particular question: plasmids R91 and R91a were both present in an isolate of Pseudomonas aeruginosa and both specify carbenicillin resistance. However, they are different in other respects, including of course the incompatibility groups to which they belong. It was shown here that the carbencillin resistance is in both cases due to a DNA sequence that corresponded to that of the transposon Tn1.

(30) S. A. Bayley, D. Morris and P. Broda. The relationships of degradative and resistance plasmids of Pseudomonas belonging to the same incompatibility group. Nature 280, 338-339 (1979).

Incompatibility testing had resulted in the assignment of Pseudomonas plasmids into twelve groups. A noteworthy fact was that within the IncP9 group there was both degradative and resistance plasmids. Since these were isolated respectively from soil and hospital bacteria originally the question arose whether the DNA of the plasmids from these two classes were in fact related. Tests with two R plasmids, R2 and pMG18, and two degradative plasmids, TOL and NAH, showed that there was a small segment common to them all, which presumably carried replication functions. This type of result shows that the population of plasmids in rather different host bacteria can be connected, as does the distribution of transposons.

Section VII. (31) "Plasmids" W.H.Freeman, 1979 Viii + 197 pp.

A table of contents for this book is included, since I consider that it has had a useful role in drawing together the evidence then to hand on the role of plasmids in the evolution and biology of bacteria. It emphasises the ubiquity of plasmids, the number of examples of their relationships, and the importance of plasmids in association with translocatable elements in reflecting genetic

exchange that has resulted in evolution that can be documented. The book also illustrates the utility of plasmids in the study of such fundamental aspects of molecular biology as DNA replication and cell-cell interactions.

Statement of candidate's contribution to the work presented in this thesis

The work described in papers 1 and 2 was performed while I was a postgraduate student, and is also presented in my Ph.D. thesis. Dr. M. Masters was the senior author of paper 6 and made the larger contribution; however, this work would not have been possible without my active participation. Papers 12, 13, 14, 16 and 17 resulted from an equal collaboration with Dr. J. F. Collins, but the contribution of Drs. Williams and Worsey to papers 19 and 20 was solely in the provision of previously published strains; all the work described was done in my laboratory. Drs. Hardy and Jeenes made specific contributions to papers 20 and 26a respectively. All other papers describe work done by me alone or with students, research assistants and visiting workers only.

Genet. Res., Camb. (1964), **5**, *pp.* 489–494
With 2 text-figures
Printed in Great Britain

SHORT NOTES

The characterization of a new type of F-prime factor in *Escherichia coli* K12

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(*Received* 9 *September* 1964)

INTRODUCTION

The sex factor, F, which is harboured by donor cells of *E. coli* K12, can exist either in the autonomous state (in F^+ cells) or integrated in the chromosome (in Hfr cells). During conjugation Hfr strains transfer the chromosome to recipient (F^-) cells in an oriented fashion, the sex factor itself entering on the tail of the structure transferred. Hfr strains can revert to the F^+ state by release of their integrated sex factor from the chromosome. An integrated sex factor may also return to the autonomous state carrying with it chromosomal markers; variant sex factors of this type are termed F-prime factors.

Studies on several F-prime factors have indicated that they carry the segment of the chromosome transferred distally in conjugation by the ancestral Hfr strain (Jacob & Adelberg, 1959; Hirota & Sneath, 1961; Pittard, Loutit & Adelberg, 1963). In this communication we describe the characterization of a new type of F-prime factor carrying segments from both distal and proximal regions of the ancestral Hfr chromosome. A model for F-prime factor formation is presented which accounts for the properties of structures both of this type and those previously described.

METHODS AND MATERIALS

Bacterial strains. The following strains were employed: C600 *thr⁻leu⁻B₁⁻lac⁻S^r*F^- (Appleyard, 1954); an F^- derivative (isolated by Dr R. C. Clowes) of W1655 *met⁻S^s*F^+ (Lederberg & Lederberg, 1953); J62 *pro⁻try⁻his⁻lac⁻S^s*F^- (Clowes & Rowley, 1954); X33 B_1*ad⁻ura⁻try⁻his⁻lac⁻S^r*F^- (supplied by Dr S. Brenner); 1177 B_1*ad⁻lac⁻S^r* (supplied by Dr P. G. de Haan); Hfr B11 (Hayes, 1964), a derivative of W1655 F^+, transferring its genes in the order *O-T6-ad-try ... pro-lac*-F; Hfr 13 (Hirota & Sneath, 1961), derived from 58–161 *met⁻S^s*F^+. This strain donates its genes during conjugation in the order *O-T6-ad-try ... pro-lac*-F. HfrH *λ⁻met⁻S^s*, which transfers its genes in the order *O-thr-leu-pro-lac-ad-try* ... F (Hayes, 1953).

Abbreviations: ad, adenine; B_1, vitamin B_1; *his*, histidine; *lac⁺/lac⁻*, ability/inability to ferment lactose; *leu*, leucine; *met*, methionine; *O*, leading extremity of an Hfr chromosome in transfer; *pro*, proline; *S^r/S^s*, resistance/sensitivity to streptomycin; *T6^r/T6^s*, resistance/sensitivity to bacteriophage T6; *thr*, threonine; *try*, tryptophan; *ura*, uracil; *λ⁺/λ⁻*, lysogenic/non-lysogenic for the temperate bacteriophage *λ*.

Media and culture methods were as described in de Haan & Gross (1962).

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RESULTS

The Hfr strain B11 (Hayes, 1964) transfers the genes for sensitivity to the phage T6 ($T6^s$) and for adenine independence (ad^+) as proximal markers during conjugation, whilst the *lac* region is transferred on the distal segment of the chromosome, and thus only enters a recipient cell after mating has been in progress for about 100 min. (Fig. 2 (A)). This property may be used to isolate F-prime factors arising spontaneously in a culture of the Hfr strain. By isolating lac^+ recombinants after mating the donor for a restricted period (60′) with a lac^- recipient, one may select recombinants which have received the lac^+ marker not by chromosomal transfer but by transfer on an F-prime factor in the autonomous state (Jacob & Adelberg, 1959).

F-prime factors of this type were isolated by mating a young broth culture of the streptomycin-sensitive Hfr strain with a similar culture of the recipient strain C600 $thr^-leu^-B^-lac^-T6^sS^r$. After an hour the mixture was violently shaken to separate mating pairs and diluted 1000-fold into broth containing streptomycin (250 μg./ml.) to kill the donor strain. The treated mixture was grown overnight at 37° C. to allow epidemic spread of the autonomous F-prime particles through the recipient population. After incubation, dilutions of the culture were spread on EMB lactose medium containing streptomycin. On this medium lac^+S^r recombinants appeared as papillae on a background of recipient (lac^-S^r) cells after incubation for 2 days. The recombinants were picked, purified and tested for their ability to transfer the lac^+ property to a lac^- recipient strain. In test crosses against the strain 1177 $B_1^-ad^-lac^-T6^rF^-$ it was shown that four of the five recombinants tested transfer lac^+ with a high efficiency and were therefore concluded to harbour F-prime factors carrying the *lac* region.

Examination of the lac^+ recombinants isolated from the test crosses shows that a considerable proportion of them inherit the ad^+ and $T6^s$ markers of the donor strain. This observation suggests that the F-prime factor isolated carries not only the *lac* region of the bacterial chromosome but also the segment bearing the ad^+ and $T6^s$ genes. This conclusion is confirmed by the results of an experiment (Table 1) in which recombinants

Table 1. *The response of F-prime BB1 to acridine orange*

Two of the lac^+S^r isolates recovered after mating the recipient strain C600 $thr^-leu^-B_1^-lac^-S$ with Hfr B11 (see text) were crossed with the strain 1177 $B_1^-ad^-lac^-S^r$. One ad^+lac^+ recombinant from each cross was picked, purified and inoculated into broth and into broth containing 50 μg./ml. acridine orange at pH 7·6 to give a final inoculum of about 100 cells/ml. After incubation single colonies were reisolated on EMB-lactose medium and the lac^+ and lac^- colonies obtained were tested by replication on to appropriately supplemented minimal medium for retention of the ad^+ phenotype.

		Phenotype of colonies			
		% ad^+lac^+	% ad^-lac^+	% ad^-lac^-	Total tested
Recombinant I	Subcultured in broth	100	0	0	74
	Subcultured in broth plus acridine orange	77	7	16	92
Recombinant II	Subcultured in broth	100	0	0	37
	Subcultured in broth plus acridine orange	48	7	45	162

were tested for the effect on their lac^+ and ad^+ phenotypes of subculture in acridine orange. Such treatment leads to the loss of autonomous sex factors (Hirota, 1960). It will be observed that a large proportion (70–80%) of those cells losing their lac^+ character

after subculture in acridine orange also lose the ad^+ phenotype, providing confirmation of our conclusion that both markers are located on the F-prime factor.

Some information on the structure of this new F-prime factor (F-prime BB1) has been obtained by determining the order in which a donor strain carrying the factor transfers the ad^+ and lac^+ markers to a recipient strain.

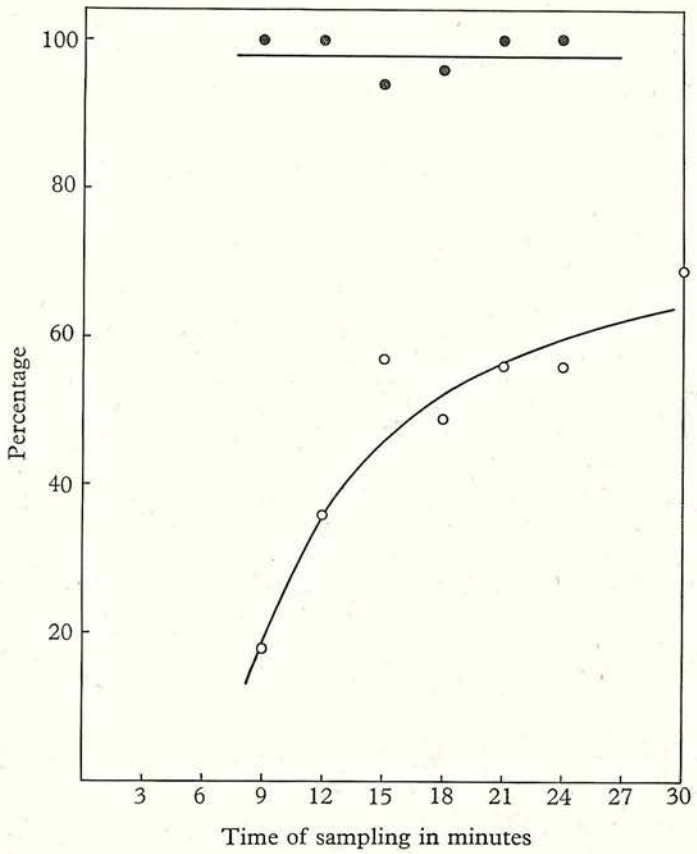

FIG. 1. The order of *ad* and *lac* on F-prime BB1. A young broth culture of W1655 *met*$^-$ *ad*$^+$ *lac*$^+$ S^s (F′BB1 *ad*$^+$ *lac*$^+$) was mixed with an equal volume of 1177 B_1^- *ad*$^-$ *lac*$^-$ S^r. At intervals samples were withdrawn, agitated to separate mating pairs and plated after dilution on to minimal-agar plates, appropriately supplemented for the selection of *ad*$^+$ S^r and *lac*$^+$ S^r recombinants. The resulting recombinant colonies were then picked, purified and tested for the *lac* and *ad* characters.

○ = percentage of *ad*$^+$ recombinants which are *lac*$^+$.
● = percentage of *lac*$^+$ recombinants which are *ad*$^+$.

A donor carrying the F-prime factor was crossed with a *lac*$^-$ *ad*$^-$ S^r recipient and the *ad*$^+$ and *lac*$^+$ recombinants obtained after increasing periods of mating were tested for inheritance of *lac*$^+$ and *ad*$^+$ respectively (Fig. 1). Fig. 1 shows that the proportion of *ad*$^+$ recombinants which are *lac*$^+$ increases with increasing time of mating while the proportion of *lac*$^+$ which are *ad*$^+$ remains constant, showing that *ad*$^+$ precedes *lac*$^+$ during transfer on the F-prime BB1 factor. Since all the available evidence (Hirota & Sneath, 1961;

Pittard, Loutit & Adelberg, 1963) indicates that the sex factor is transferred distally on an F-prime factor it is concluded that F-prime BB1 has the structure: *ad-lac*-F.

Studies on the transfer of chromosomal markers by a donor strain harbouring the new F-prime factor have been made. The frequency of transfer of the chromosomal markers pro^+ and try^+ by the strain W1655 (F′ BB1) was compared with their frequency of transfer by the ancestral Hfr strain, B11. In parallel crosses, mating young broth cultures of the two donors with the recipient strain J62 for one hour, it was found that in both cases try^+ was transferred over 100 times more frequently than pro^+, indicating that the F-prime factor causes the chromosome to be transferred with the same orientation as in the ancestral Hfr strain.

There is a clear similarity between the F-prime factor BB1 and the F-13 factor of Hirota & Sneath (1961). Both structures carry the chromosomal markers *T6*, *ad* and *lac*. Kinetic studies have shown that *ad* precedes *lac* in transfer on both structures (see Fig. 1) and that the entry times for these two markers correspond in the two F-prime factors. In addition, the results presented in Table 2 indicate that the sex factors of the two

Table 2. *The transfer of* ad^+ *by Hfr 13*

Young broth cultures of the Hfr strains B11, 13 and H were mated in parallel crosses against strain X33 B_1^- *ad-ura-try-his-lac-S*^{r}F$^-$ for 1 hour. Dilutions were plated on minimal medium appropriately supplemented for the selection of try^+ recombinants. Colonies were purified on the same selective medium and tested for the *ad* character.

Donor strain	% try^+ which were ad^+	No. of try^+ recombinants tested
Hfr B11	36	194
Hfr 13	57	102
Hfr H (control)	32	79

parental Hfr strains are integrated at closely similar sites. Try^+ recombinants recovered from parallel crosses using Hfr B11 and Hfr 13 as donors were scored for inheritance of the ad^+ marker from the donor. It was expected that if Hfr 13 transfers ad^+ as a proximal marker the proportion of try^+ recombinants inheriting ad^+ would be essentially the same as in the Hfr B11 cross, whereas if Hfr 13 transfers ad^+ as a distal marker virtually none of the try^+ recombinants should be ad^+. The results presented in Table 2 show that Hfr 13 does transfer ad^+ as a proximal marker, leading to the conclusion that the two Hfr strains must both carry their sex factors integrated between *ad* and *lac*.

DISCUSSION

Figure 2 (A–C) shows a formal representation of the events postulated for the formation of an F-prime factor. Firstly, breaks occur at the sites labelled x and y in the figure. Secondly, rejoining of the ends occurs in such a way as to (a) restore the circularity of the bacterial chromosome and (b) give rise to a closed structure, the F-prime factor, which has transfer properties directly analogous with the ancestral Hfr chromosome (Scaife & Gross, 1964).

In Fig. 2 (D) we present a model which would incorporate the above two stages in a single event. If the sites x and y represent regions of limited genetic homology, synapsis between them, followed by a reciprocal genetic exchange at the site of pairing would give rise to the two structures shown in Fig. 2 (C).

The model proposed has the following predictions. Firstly the chromosomal markers of the Hfr strain incorporated into the F-prime factor should retain their original

orientation of transfer during conjugation. Our observation that the marker ad^+ precedes lac^+ on F-prime BB1 confirms this prediction. In addition, preliminary results indicate that the $T6^s$ marker on F-prime BB1 enters recipient cells before ad^+, as expected on the above model. Secondly, it is predicted that the chromosome in the cell where the

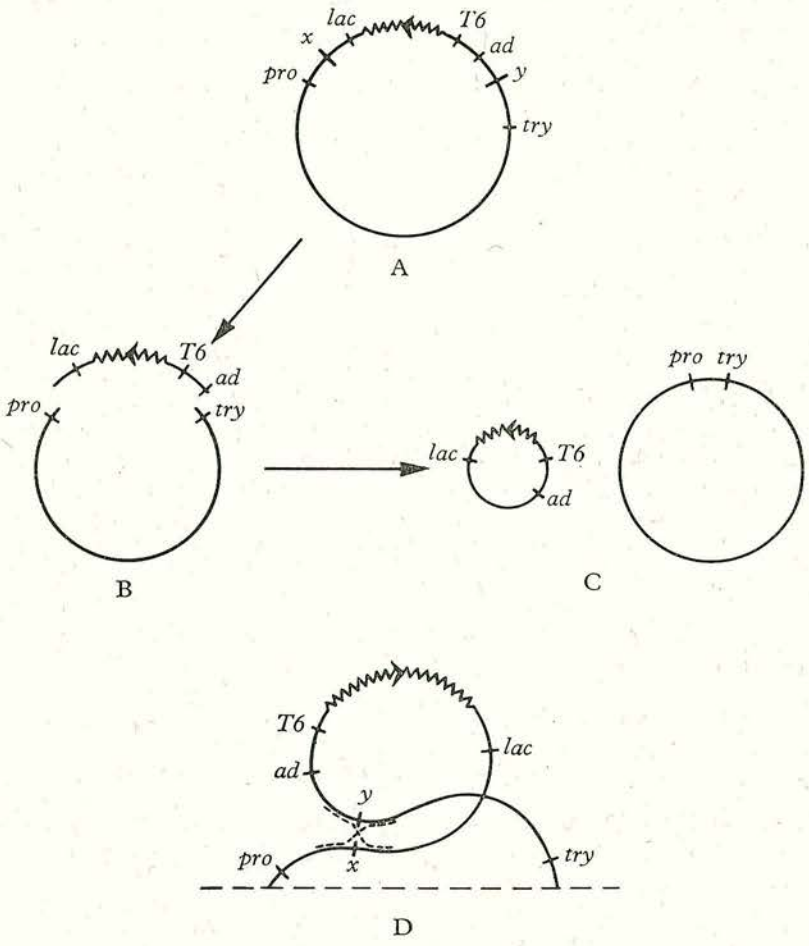

FIG. 2. The mechanism of F-prime formation in *E. coli*. Diagrams A, B, and C represent formally the sequence of events necessary for the formation of F′ BB1 (see text). A → B shows the release of the F factor associated with a piece of the bacterial chromosome. In B → C there is union of the open ends of the resulting structures.

Diagram D shows the model proposed. By assuming a genetic exchange between sites x and y (see text) the above sequence can be expressed as a single event.

The sex factor, represented by a wavy line, is shown inserted in the chromosome (Campbell, 1962). The direction of transfer during conjugation is indicated by the arrow.

F-prime factor arose should have a deletion corresponding to the fragment contained in the new structure. Evidence presented in a separate report (Scaife & Pekhov, 1964) indicates that this is the case. Thirdly, we expect that any Hfr strain with its sex factor

integrated at a site between the homologous regions x and y could give rise to F-prime factors carrying both *ad* and *lac*. Our results indicate a close similarity between Hfr 13 and Hfr B11. The observation (Hirota & Sneath, 1961) that F13 carries the ad^+ and lac^+ markers is therefore consistent with the above model.

It may be pointed out that the model presented will account for the formation of F-prime factors not carrying the proximal region of the parental Hfr chromosome if it is assumed that the site corresponding to y in Fig. 2 may lie within the sex factor itself. An analogous genetic exchange involving such a site would result in the retention of a fragment of the sex factor by the bacterial chromosome. Such a fragment would correspond to the sex factor attachment locus observed by Adelberg & Burns (1960) and by Richter (1961).

REFERENCES

Adelberg, E. A. & Burns, S. N. (1960). Genetic variation in the sex factor of *E. coli*. *J. Bact.* **79**, 321–330.

Appleyard, R. K. (1954). Segregation of new lysogenic types during growth of a double lysogenic strain derived from *Escherichia coli* K12. *Genetics*, **39**, 440–452

Campbell, A. M. (1962). Episomes. *Advanc. Genet.*, **11**, 101–145.

Clowes, R. C. & Rowley, D. (1954). Some observations on linkage effects in genetic recombination in *Escherichia coli* K-12. *J. gen. Microbiol.* **11**, 250–260.

De Haan, P. G. & Gross, J. D. (1962). Transfer delay and chromosome withdrawal during conjugation in *E. coli*. *Genet. Res.* **3**, 251–272.

Hayes, W. (1953). The mechanism of genetic recombination in *Escherichia coli* K12. *Cold Spr. Harb. Symp. quant. Biol.* XVIII, 75–93.

Hayes, W. (1964). *The Genetics of Bacteria and their Viruses*. Blackwell.

Hirota, Y. (1960). The effect of acridine dyes on mating type factors in *E. coli*. *Proc. nat. Acad. Sci.*, Wash., **46**, 57–64.

Hirota, Y. & Sneath, P. H. A. (1961). F′ and F-mediated transduction in *Escherichia coli* K12. *Jap. J. Genet.* **36**, 307–318.

Jacob, F. & Adelberg, E. A. (1959). Transfert de caracteres genetiques par incorporation au facteur sexuel d'*Escherichia coli*. *C. R. Acad. Sci., Paris*, **249**, 189–191.

Lederberg, E. & Lederberg, J. (1953). Genetic studies of lysogenicity in *E. coli*. *Genetics*, **38**, 51–64.

Pittard, J., Loutit, J. S. & Adelberg, E. A. (1963). Gene transfer by F′ strains of *E. coli* K12. I. Delay in initiation of chromosome transfer. *J. Bact.* **85**, 1394–1401.

Richter, A. (1961). Attachment of wild-type F factor to a specific chromosomal region in a variant strain of *E. coli* K12; the phenomenon of episomic alternation. *Genet. Res.* **2**, 335–345.

Scaife, J. G. & Gross, J. D. (1964). The integration of episomes into the bacterial chromosome, in *Struktur und Funktion des genetischen Materials* (Erwin-Baen-Gedachtnis vorlesnagh III, 1963) Abhandlungen d.D.A.d.w., Klasse f. Medezin, No. 1. Ed. Stubbe, H. In press.

Scaife, J. G. & Pekhov, A. (1964). Deletion of chromosomal markers in association with F-prime factor formation in *E. coli* K12. *Genet. Res.* **5**, 495–498.

Genet. Res., Camb. (1967), 9, pp. 35–47
With 1 text-figure
Printed in Great Britain

The formation of Hfr strains in *Escherichia coli* K12

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(*Received* 19 *May* 1966)

1. INTRODUCTION

The work described in this paper was undertaken to establish whether in a typical F^+ strain of *E. coli* K12 stable Hfr strains arise with their origins (that is, the points at which the sex factor is integrated) distributed randomly around the chromosome, or whether there are specific regions at which this integration may occur. A single F^+ strain was used as a source of independently and spontaneously arising Hfr strains. The analysis of these supports the view that there exist specific regions for sex factor integration. Further experiments bearing on the mechanism of formation of Hfr strains and on the nature of the fertility of F^+ cultures are also described.

2. MATERIALS AND METHODS

(i) *Bacterial strains*

The following strains of *E. coli* K12 were used:

W1655 F^+ *met*$^-$ *str*^{s} *T6*^{s} *lac*$^+$ *gal*$^+$ $(\lambda)^-$ λ^r (Lederberg & Lederberg, 1953).

W677 F^- *thr*$^-$ *leu*$^-$ B_1^- *pro*$^-$ *str*^{r} *azi*^{s}. A *str*^{r} derivative, kindly provided by Dr R. C. Clowes, of a *pro*$^-$ derivative of strain W677 (Hayes, 1953).

W677 F^- *thr*$^-$ *leu*$^+$ B_1^- *pro*$^-$ *str*^{r} *azi*^{s}. The *leu*$^+$ marker from strain W1655 was introduced into the previous strain, using phage P1*kc* (Lennox, 1955) as the transducing agent.

W945 F^- *thr*$^-$ *leu*$^-$ B_1^- *str*^{r} *T6*^{r} *lac*$^-$ *gal*$^-$ (Cavalli-Sforza & Jinks, 1956).

Hfr P4X *met*$^-$ *str*^{s} *lac*$^+$ *gal*$^+$ (Adelberg & Burns, 1960).

J62 F^- *pro*$^-$ *try*$^-$ *his*$^-$ *str*^{r} *lac*$^-$ (Clowes & Rowley, 1954).

1177 F^- *ade*$^-$ B_1^- *str*^{r} *T6*^{r} *lac*$^-$ (a *T6*^{r} derivative of a strain provided by Dr P. G. de Haan).

The origins and directions of transfer of the Hfr strains mentioned in the text, as well as the symbols for the genetic markers, are given in Fig. 1.

(ii) *Media*

Nutrient broth: 2·5% Oxoid Nutrient Broth No. 2.

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Minimal media; as described by Lederberg & Tatum (1947) but without asparagine.

Nutrient and minimal agar were made by solidifying the appropriate liquid media with 1·25% and 1·5% Davis New Zealand Agar respectively.

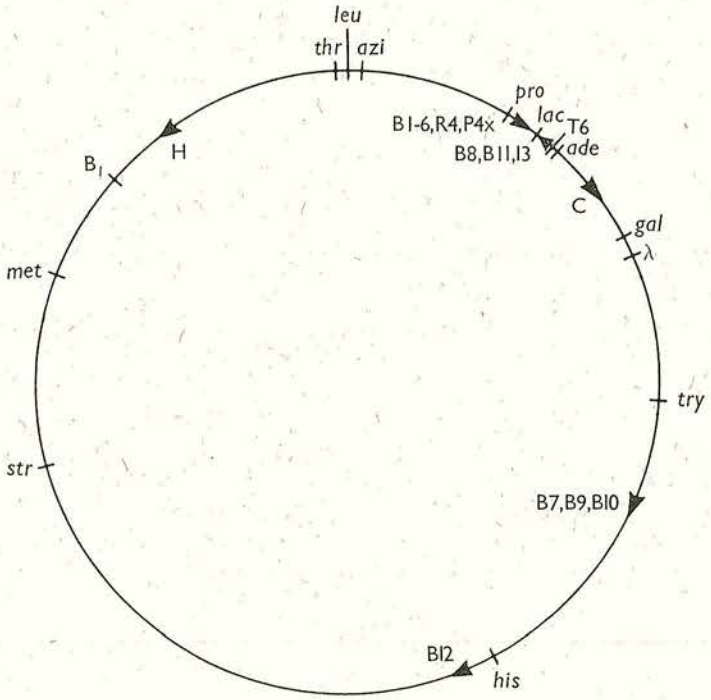

Fig. 1. The genetic map of *Escherichia coli* K12, showing the location of markers mentioned in this paper. The arrows represent the origins and directions of transfer of Hfr strains mentioned in the text. Markers are shown outside the circle and Hfr strain symbols inside. The abbreviations used are the following.

ade adenine, B_1 vitamin B_1, *his* histidine, *leu* leucine, *met* methionine, *pro* proline, *thr* threonine, *try* tryptophan, λ = prophage λ. *azi*, *str*, *T6*; resistance/sensitivity to azide, streptomycin, and phage *T6* respectively. *gal*, *lac*; fermentation of galactose, lactose.

The origins of the following Hfr strains are given: C (Cavalli, 1951); H (Hayes, 1953); P4X (Adelberg & Burns, 1960); R4 (Reeves, 1959); 13 (Hirota & Sneath, 1961); B1–12 (see text).

Buffer: An aqueous solution (pH approx. 7·2) with 0·7% anhydrous $NaHPO_4$, 0·4% NaCl, 0·3% KH_2PO_4 and 0·02% $MgSO_4.7H_2O$.

Aminoacids, vitamin B_1 and streptomycin were added to give final concentrations of 20, 10 and 250 μg./ml. respectively.

(iii) *Culture methods*

Strains were maintained at 4°C. on nutrient agar slopes. All experiments were performed at 37°C. Stationary-phase cultures were obtained by inoculating into

nutrient broth and incubating without aeration. After overnight growth such cultures contain about 10^9 cells per ml.

(iv) *Interrupted mating experiments*

In interrupted mating experiments a hundred-fold dilution of an overnight culture of the donor strain was made into fresh broth. This culture was incubated for $1\frac{3}{4}$ hours (giving about 2×10^7 cells per ml.) on a 33 r.p.m. rotor, at 37°C., before mixing with an equal volume of an overnight culture of the recipient strain. At appropriate times samples were withdrawn, diluted ten-fold into buffer, violently agitated for 1 min. on a Microid flask shaker running at full speed, and further diluted five-fold. Samples (0·2 ml.) were plated onto appropriate selective media.

(v) *Technique for the isolation of Hfr strains*

An overnight culture of the F+ strain W1655 *met*$^-$ *str*^{s}, derived from a single colony, was diluted into fresh broth to give a suspension with about 100 cells per ml. Forty-four 1 ml. aliquots were dispensed into tubes, which were then incubated at 37°C. for $5\frac{1}{2}$ hours, to give populations of about 5×10^6 cells per ml. Samples (0·15 ml.) were withdrawn and mated for 1 hour at 37°C. with 0·1 ml. of a culture of an F$^-$ strain (about 2×10^7 cells) suspended in buffer. A drop of this mixture was then plated onto selective medium. The remainder of the F+ cultures were stored at 4°C. until it was known which of them would be used for the next cycle of the enrichment procedure.

The cultures which gave rise to many recombinant colonies were inferred to contain a large clone of Hfr cells with an origin relatively close to the selected markers. Such cultures were only enriched if they gave more than four times the average number of recombinants; they were then diluted into fresh broth, so that there was about one Hfr cell per 10 ml. The number of recombinants obtained in the above cross was used as the approximate, empirical, dilution factor. One millilitre aliquots were then dispensed into a fresh series of tubes and incubated for $4\frac{1}{2}$ hours before mixing 0·15 ml. samples with the female strain, and plating as before for recombinants. In the isolation of the different Hfr strains, one to three cycles of dilution, incubation and mating were required before the cultures were sufficiently enriched to allow plating on nutrient agar for the picking of Hfr colonies. This process was repeated to obtain each Hfr strain isolated.

(vi) *Rate of reversion from the Hfr to the F+ state*

Overnight broth cultures of each of these strains were diluted $\times 2{\cdot}10^{-10}$ into broth and dispensed as 1 ml. aliquots so that the initial number of cells in any one tube is unlikely to be more than one. After overnight incubation those tubes which yielded

growth were checked for fertility in crosses with appropriate recipient strains and dilutions were plated for the recovery of isolated colonies. One hundred colonies derived from each Hfr strain were resuspended in 1 ml. of broth, and 0·5 ml. portions were used for scoring fertility of the Hfr or the F^+ type by mixing with an equal volume of a culture of an F^- strain and after 3 hours' incubation plating standard loopfuls (*ca.* 0·02 ml.) of the mixture for recombinant colonies on selective media. This technique gives better discrimination than the use of replica plating. In general each clone gave a clear Hfr or F^+ level of fertility. The few clones which were doubtful, and all apparent F^+ clones, were retested to ensure that low fertility was not an artefact.

3. RESULTS

(i) *Isolation and characterization of Hfr strains*

Hfr strains were isolated by a modification of the method of Jacob & Wollman (1956). These authors obtained F^+ cultures containing a high proportion of Hfr cells by the Luria and Delbrück fluctuation method (1943), and isolated Hfr colonies from such cultures by replica plating. In the present study, the method (see Materials and Methods, Section (v)) differs in three respects. First, no ultraviolet irradiation was used. Second, cells were kept in broth at all stages of the isolation procedure, so that mutations to auxotrophy which might accompany the transition from the F^+ to the Hfr state would not necessarily be lethal. Third, Hfr strains were isolated from the original Hfr-rich F^+ cultures by Cavalli and Lederberg's sib-selection enrichment technique (1955) instead of by replica plating. This involves estimating how many Hfr cells there are in a culture so that when aliquots from a dilution of this culture are dispensed into a series of tubes only a few of them will receive an Hfr cell. In this minority of cultures the Hfr population will therefore be enriched with respect to the background of F^+ cells because the ratio of F^+ cells to Hfr cells in these few tubes will be smaller than in the original undiluted culture. This method has been used independently by Mäkelä (1963) for the isolation of Hfr strains in *Salmonella abony*. Each Hfr strain isolated was derived from a culture started from a single F^+ colony; there was therefore no possibility of the repeated isolation of the same Hfr clone.

The strain used in this study was W1655 F^+, a methionineless, non-lysogenic derivative of the original Lederberg (1947) strain 58-161. In matings it gives approximately equal numbers of recombinants for markers distributed around the chromosome, so that there is no special affinity of the sex factor for any particular region of the chromosome like that described by Richter (1961). In the first series of isolations, selection was for Hfr strains which transfer thr^+leu^+ early; the recipient strain used was W945 $thr^-leu^-B_1^-str^r$. Six independently arising Hfr strains were obtained; these were characterized by 1 hour crosses and by interrupted matings (see Methods), in which it was found that each of these strains (B1–6) began to transfer the pro^+ and thr^+leu^+ genes at about $5\frac{1}{2}$ and 13 min. mating respectively.

None of them transferred the *lac*$^+$ locus with high efficiency. It was therefore concluded that the origins of all these strains are in the small region between *pro* and *lac*. This result strongly suggests that there are specific regions where the sex factor can integrate stably to give Hfr strains.

To determine whether another class of Hfr strains exists in which the sex factor is also integrated between the *pro* and *lac* loci, but which transfer the chromosome in the opposite direction, a second series of isolations was undertaken. The recipient strain used was J62 (*pro*$^-$*try*$^-$*his*$^-$*str*^{r}), and the isolation of Hfr strains transferring the *try*$^+$ gene (which is about the same distance as the *thr leu* loci from the *pro-lac* region) was attempted. Six further Hfr strains (B7–12) were isolated; they fell into three groups. Whereas strain B12 transferred *his*$^+$ very early, the origins of strains B8 and B11 fell between the *T*6 and *lac* loci; these two strains, which have very similar transfer kinetics, transfer *ade*$^+$ early and *lac*$^+$ late, and were found to be very similar to Hfr strain 13 (Hirota & Sneath, 1961). Hfr strains B7, 9 and 10 form the third class. All three transfer *try*$^+$ after 7–10 min. and *his*$^+$ late. However, since Hfr strains with the sex factor integrated between *pro* and *lac*, transferring *lac* first, were not found, it was tentatively concluded that in this region at least, the sex factor can only integrate to give Hfr strains transferring the chromosome with one polarity.

(ii) *To distinguish between apparently similar Hfr strains*

(*a*) *Transduction*

An obvious approach was to compare the linkage between the sex factor and the closest known chromosomal markers, using transduction with phage P1*kc* (Lennox, 1955). In such an experiment, using Lennox's methods, Hfr strain B1 and a *pro*$^-$*lac*$^-$ strain were used as the donor strain and the recipient strain respectively. The recipient strain was a *pro*$^-$ derivative of strain W677 *thr*$^-$*leu*$^-$$B_1^-$ made *leu*$^+$ so that a marker transferred early by Hfr strain B1 was available for the testing of *lac*$^+$ transductants for the Hfr property. Although both *pro*$^+$ and *lac*$^+$ transductants were obtained, of several hundred recombinants tested, none had inherited either the unselected marker (that is, *lac*$^+$, *pro*$^+$ respectively) or the sex factor.

Since DeWitt & Adelberg (1962) had reported joint transduction of the *lac*$^+$ character and the sex factor in an experiment in which Hfr strain P4X was used as the donor and a recipient strain analogous to strain W677 *pro*$^-$ as the recipient, Hfr strain P4X was then used in a control experiment. Again, none of the *lac*$^+$ transductants obtained carried the sex factor. Because of the absence of any positive result, this approach was not pursued.

(*b*) *The rate of reversion from the Hfr to the F*$^+$ *state*

It was thought that each species of Hfr strain might have a characteristic instability. The proportion of F$^+$ cells among the descendants, in a known number of generations, of single cells of the different Hfr strains was therefore determined and compared (see Methods).

It was found that with none of the Hfr strains tested (B1–11) had the proportion of F^+ bacteria became greater than 2% after the thirty generations of growth from one cell to about 10^9 cells. Therefore, to obtain greater resolution, clones which had been serially subcultured through a total of about eighty generations were examined. It was important to show that the results obtained by this method with a given Hfr strain are reproducible. All determinations were therefore made on two or three parallel cultures, each starting from a different single cell of the Hfr strain.

Table 1. *The formation of F^+ derivatives during subculture of Hfr strains B1–11*

Hfr strain	Approximate number of generations of growth	Number of colonies tested	Number of revertant clones
B1	80	200	3
B2	80	100	0
B3	80	100	0
B4	80	100	0
B5	80	100	0
B6	80	100	0
B7	30	100	0
	80	100	91
B9	30	100	2
	80	100	96
B10	30	100	0
	80	100	99
B8	80	100	1
B11	80	100	3

Overnight broth cultures of Hfr strains B1–11 were diluted $2 \cdot 10^{-10}$ into broth and dispensed as 1 ml. aliquots. After overnight incubation, dilutions were made, first 2×10^6 into broth for a second cycle of overnight growth, and, second, for plating on nutrient agar for the recovery of 30-generation colonies. After three cycles of dilution and overnight incubation, dilutions and platings were made for the recovery of 80-generation clones. Colonies were tested for the Hfr character by suspending in broth, incubating and crossing with appropriate F^- strains (W677 *thr⁻leu⁻B₁⁻pro⁻str^r* for strains B1–6, and J62 *pro⁻try⁻his⁻str^r* for strains B7–11).

Standard loopfuls (*ca.* 0·02 ml.) of the mating mixtures were plated on to selective media for the recovery of *thr⁺leu⁺* (strains B1–6) and *try⁺* (B7–11) recombinants. In each case the results given are the sum of two or three parallel experiments.

It was confirmed that within an Hfr strain the data from parallel experiments are very similar. For example, in the experiment given in Table 1 the three Hfr B7 clones subcultured in parallel gave rise to 32/34, 30/33 and 29/33 revertants.

The results are presented in this table. Hfr strains with similar origins tended to give similar numbers of F^+ cells, but Hfr strains with different points of origin yielded very diverse numbers of F^+ cells. Thus Hfr B8 and B11 resembled each other, as did strains B7, 9 and 10. An exception was strain B1. Two out of three subcultures of this strain gave rise to F^+ revertants. It therefore appears to differ from strains B2–6, if these are considered together. No revertants were found among any subcultures of these strains, so that they are indistinguishable within the limits of the experiment.

A very interesting point emerges from a comparison of the thirty- and eighty-generation samples from strains B7, 9 and 10. It appears from these data that during growth of these strains the proportion of F^+ cells increases exponentially. The simplest explanation, assuming a constant rate for the Hfr→F^+ transition, is that the Hfr cells grow more slowly than the F^+ cells. To account for the observed rate of increase of the F^+ population in these cultures the Hfr cells would have to grow at about 0·76 of the rate (1·0) for F^+ cells. The growth rates of strains Hfr B10 and W1655 F^+ in broth at 37°C., measured by dilution and plating for viable colony counts, were compared; the experimental result obtained was 0·79. We may therefore conclude that there is a difference in growth rate, sufficient to explain this effect, between Hfr strains of the B7 type and the F^+ strain from which they arose, and that the latter grows at a similar rate to the F^+ revertants. However, slower growth rate is not a general property of Hfr strains, since strains W1655 F^+ and Hfr B1 grow at the same rate.

(c) *The transfer race technique*

The object of this refinement of the interrupted mating experiment (suggested by Dr Julian Gross) is to exclude some of the factors which cause fluctuations between the results of different experiments. Comparisons between pairs of Hfr strains are made by using them in a mixed mating with the same F^- strain, so that all sampling errors and environmental effects cancel out. The contribution which each of the two donor strains makes to the recombinant population can be assessed by labelling one of them with a non-selective marker which is inherited with a high and constant frequency by the selected recombinants. In the present study the entry times of *thr*$^+$*leu*$^+$ for Hfr strains B1 and B3 were compared, since although they were indistinguishable in simple interrupted mating experiments, in the reversion experiment (see above) only strain B1 gave F^+ derivatives at a measurable rate. Azide resistance (azi^r) was used as the marker labelling one of the Hfr strains; since both strains transfer their *azi* allele just before *thr*$^+$*leu*$^+$, all the selected recombinants will have received the *azi* locus, irrespective of the time at which mating is interrupted. If one of the strains transfers the *thr*$^+$*leu*$^+$ loci earlier than the other strain does, in mixed matings the 'winning' strain will contribute a higher proportion of the first-formed recombinants; these can be recognized by the *azi* allele which they carry.

Azide-resistant derivatives of strains B1 and B3 were isolated and the following pairs of strains were together mated with the female strain W677 *thr*$^-$*leu*$^-$*B*$_1^-$*pro*$^-$*str*^{r}*azi*^{s}

B3 *azi*^{s} + B3 *azi*^{r}

B3 *azi*^{s} + B1 *azi*^{r}

The former cross (Table 2) showed that the *azi* character itself did not affect the kinetics of mating. The result of the second cross shows that within the limits of the

experiment, Hfr strains B1 and B3 are indistinguishable with respect to the time of transfer of the *thr*$^+$ and *leu*$^+$ loci.

Table 2. *The transfer-race experiment*

Hfr strains in cross	Time at which mating is interrupted	Total number of *thr*$^+$*leu*$^+$ recombinants obtained per 0·2 ml. sample	Number of recombinants tested	*azi*^{r}	
				No.	%
B3 *azi*^{s} + B3 *azi*^{r}	12	96	108	71	66
B3 *azi*^{s} + B3 *azi*^{r}	25	712	108	66	61
B3 *azi*^{s} + B1 *azi*^{r}	12	150	72	47	65
B3 *azi*^{s} + B1 *azi*^{r}	25	> 1000	72	47	65

Overnight broth cultures of the Hfr strains were diluted 10^{-2} into fresh broth. After $1\frac{3}{4}$ hours' incubation at 37°C. on a rotor the two Hfr cultures were mixed, and a 5 ml. volume of the mixture was added to an equal volume of an F$^-$ strain (W677 *thr*$^-$*leu*$^-$*B*$_1$$^-$*pro*$^-$*str*^{r}*azi*^{s}). At appropriate times samples were withdrawn, diluted tenfold into buffer, violently agitated for 1 min. and further diluted fivefold. Samples (0·2 ml.) were plated on minimal medium supplemented with glucose, proline, vitamin B_1 and streptomycin, for *thr*$^+$*leu*$^+$ recombinants. Recombinant colonies were resuspended in buffer and streaked on plates of the selective medium supplemented with M/1600 (final concentration) sodium azide. Under these conditions an unambiguous resistance/sensitivity result was obtained for the *azi* character.

(iii) *The analysis of recombinants formed in F*$^+$ *crosses*

In the present study selection at each stage in the isolation procedure of the tube giving rise to most recombinants, resulted in the enrichment and isolation of Hfr strains, so that we may conclude that these strains are indeed derived from the observed fertile clones. However, there is no proof that such clones are the sole basis for the fertility of F$^+$ cultures. We may ask whether Hfr strains such as those isolated (B1–12) in the present study are sufficient to explain the fertility of strain W1655 F$^+$.

Strains B1–12 divide into three groups with respect to the transfer of the *pro lac try* region of the chromosome. While strains B1–6 transfer *pro*$^+$ but not *lac*$^+$ or *try*$^+$ with high frequency, and strains B8 and B11 transfer only *try*$^+$, strains B7, 9 and 10 and also strain B12 transfer all three loci with high frequency. Thus whereas *pro*$^+$ and *try*$^+$ are transferred by two of the three groups of strains, *lac*$^+$ is only transferred by the B10 group. Therefore, if F$^+$ fertility is due to Hfr clones (and assuming that strains B1–12 are a representative sample of these), we should expect that in F$^+$ × F$^-$ crosses *lac*$^+$ will be transferred less frequently than either *pro*$^+$ or *try*$^+$ and also that the proportion of the *lac*$^+$ recombinants which are *pro*$^+$ will be greater than the proportion of *pro*$^+$ recombinants which are *lac*$^+$.

Strain W1655 F$^+$ (from which strains B1–12 had been isolated) was mated with strain J62 F$^-$ *pro*$^-$*try*$^-$*his*$^-$*lac*$^-$*str*^{r} in a 1-hour cross. Dilutions were plated onto selective media for *pro*$^+$ recombinants on the one hand, and *lac*$^+$ recombinants on the other. It was not possible to draw conclusions from the analysis of the *lac*

recombinants, since it was found that all these, both in this cross and the control Hfr cross (see below), were *pro*$^+$ and *try*$^+$. This phenomenon, also observed by Professor B. A. D. Stocker (personal communication), has been termed by him 'inadvertent selection'. However, the frequency of inheritance of the *lac*$^+$ character can be examined among the *pro*$^+$ recombinants, which were also tested for the *try* character.

Table 3. *Linkage between* pro, lac *and* try *among* pro$^+$ *recombinants in crosses of strains Hfr B*10* *and W*1655 *F*$^+$ *against strain J*62 pro$^-$try$^-$his$^-$lac$^-$str^r

1. Approximate number of recombinants per ml. of mating mixture

Cross	Recombinant class	
	pro$^+$	*try*$^+$
Hfr B10 × F$^-$	$9{\cdot}4 \times 10^6$	$3{\cdot}4 \times 10^7$
F$^+$ × F$^-$	$1{\cdot}8 \times 10^4$	$2{\cdot}8 \times 10^4$

2. Analysis of *pro*$^+$ recombinants

Donor strain	Number of recombinants tested	Percentage of the different classes of recombinants			
		lac$^+$*try*$^+$	*lac*$^+$*try*$^-$	*lac*$^-$*try*$^+$	*lac*$^-$*try*$^-$
Hfr B10	160	53	28	9	10
F$^+$	232	11	53	1	35

Logarithmically growing broth cultures were mated for 1 hour before dilution and plating on minimal selective medium for *pro*$^+$ and *try*$^+$ recombinants. Purified *pro*$^+$ recombinant colonies were suspended in buffer and streaked onto selective media to score for the *try* and *lac* characters.

* Hfr strain B10 transfers its markers in the order *try*$^+$*lac*$^+$*pro*$^+$.

A control cross was made with Hfr strain B10, which transfers genes in the order 0 *try lac pro*. It was found that of the *pro*$^+$ recombinants 81% were *lac*$^+$ and 62% were *try*$^+$ (Table 3). In the F$^+$ cross 64% of the *pro*$^+$ recombinants were also *lac*$^+$. Since linkage between these genes (as determined in the Hfr cross) is 81%, in the F$^+$ cross, in about 79% (that is, $64 \times \frac{100}{81}$) of the *pro*$^+$ recombinants the *lac*$^+$ gene will have entered the recipient cell.* Since Hfr strains B1–6 do not transfer *lac*$^+$, according to these results only $100 - 79 = 21\%$ of the *pro*$^+$ colonies can possibly have come from this type of strain.

It might be argued that Hfr clones of the B10 type would account for the *pro*$^+$ recombinants which are *lac*$^+$. However, only 12% of the *pro*$^+$ recombinants from the F$^+$ cross are *try*$^+$. Since Hfr strain B10 showed 62% linkage between the *pro*$^+$ and *try*$^+$ markers, the maximum proportion of the *pro*$^+$ recombinants which could

* It is assumed that the sizes of the DNA fragments transferred in the F$^+$ and Hfr matings are similar. If this assumption is false, it follows that F$^+$ fertility cannot be due to Hfr clones.

have been derived from this type of donor is $12 \times \frac{100}{62} = 19\%$. We must therefore conclude that less than half of the *pro*$^+$ recombinants in F$^+$ crosses originated from Hfr cells of the B1 and B10 types.

4. DISCUSSION

When Hfr strains transferring the *thr*$^+$ and *leu*$^+$ loci as proximal markers were selected for, all six isolates (strain B1–6) were found to have their origins in a small chromosomal region between *pro* and *lac*, about 10 min. of transfer time away from the *thr leu* region. The length of this *pro lac* segment is about 2% of that of the whole chromosome (Jacob & Wollman, 1961). A total of at least twelve Hfr strains transferring *pro* early and *lac* late have now been isolated. As well as the six Hfr strains B1–6 and three more strains isolated by the present writer using a replica plating method, Hfr strains R2 and R4 (Reeves, 1959) and P4X (Adelberg & Burns, 1960) also have the sex factor integrated in this region. A firm conclusion is therefore that Hfr strains isolated by this method arise by non-random integration of the sex factor into the chromosome. This conclusion has also been drawn by Sanderson & Demerec (1965) (for Hfr strains in *Salmonella typhimurium*) and Matney *et al.* (1964).

In simple interrupted mating experiments the origins of Hfr strains B1–6 were indistinguishable. In more refined transfer experiments Hfr strains B1 and B3 could still not be distinguished, although these strains differed in that F$^+$ derivatives were found in sub-cultures of strain B1 but not in those of strain B3. Unsuccessful attempts were also made to compare the linkage of the sex factors of Hfr strains B1–6 to neighbouring chromosomal genes by transduction using phage P1.

The isolation of other Hfr strains (B7–12) is also described; the fact that five out of six fall into two groups is strong support for the proposition that there are specific small regions where the sex factor can integrate to give rise to stable Hfr strains. Hfr strains of the reciprocal type to B1–6 (that is, strains with the sex factor integrated in the same region but transferring the chromosome in the opposite direction) were looked for but not found; this is evidence that these integration sites have polarity.

The repeated isolation of similar types of Hfr strain, and the absence of the reciprocal type to strains B1–6 would both be expected if the formation of Hfr strains is dependent on pairing between homologous regions of the DNA of the sex factor and of the chromosome. Models postulating that the sex factor can integrate with equal ease at all points on the chromosome are excluded, as are those which propose that the sex factor associates with non-DNA regions of the chromosome, unless assumptions on the nature of this attachment are made to account for the observed polarity. These results are consistent with Campbell's model (1962) for insertion by recombination, although they do not exclude pairing without recombination as a mechanism for integration.

In experiments with Hfr strains B1–11 cultures were grown for a similar number of generations from a single fertile cell. Striking differences were observed in the

frequencies of F^+ cells in the sub-cultures of the different Hfr strains, although in general the strains within a group gave comparable results; thus strains B7, B9 and B10 gave values respectively of 91%, 96% and 99% F^+. An exception was strain B1, which gave revertants and appeared to differ significantly in this respect from strains B2–6 which yielded none.

However, further investigation showed that the high proportion of F^+ cells in the cultures of Hfr strains B7, 9 and 10 was in each case mainly due to the fact that these Hfr strains grew much more slowly than the F^+ cells arising from them rather than to an inherently high degree of instability. This result suggests that the growth rate of an Hfr strain is a function of the region at which the sex factor is integrated.

Jacob and Wollman's observation (1956) that persistent fertile clones arise in F^+ populations has been confirmed in the present study by the isolation of Hfr strains B1–12 following enrichment from F^+ cultures chosen for their fertility. However, the examination of recombinants obtained in an $F^+ \times F^-$ cross, using the F^+ strain from which the Hfr strains were isolated, gave very different frequencies for the inheritance of unselected markers from those predicted on the assumption that F^+ fertility is due solely to Hfr strains of the types isolated in the present study. Analysis showed that Hfr clones of the B1 and B10 types together cannot contribute more than half the *pro*$^+$ recombinants obtained in the F^+ cross. It is therefore inferred that the types of Hfr strain actually isolated can only contribute a component to F^+ fertility. However, we must treat this conclusion with some caution, because if a large number of Hfr clones arose similar in transfer properties to the Hfr Cavalli strain (that is, transferring both *lac*$^+$ and *pro*$^+$) this type of result would be obtained.

A Campbell-type model for the formation of Hfr strains by recombination allows us to propose an explanation for this result, as well as for the observation that Hfr strains B7, 9 and 10 grow more slowly than their F^+ revertants and the strain from which they arise. If recombination between sex factor and chromosome occurs at non-allelic regions of limited homology distributed randomly around the chromosome, insertion within a gene or operon could lead to impairment of function and the consequent slow growth rate or even death of the cell. Such clones with a potentially lethal integration of the sex factor might persist for a number of generations, contributing to the fertility of F^+ cultures, but would be impossible to isolate. Since the procedure employed in the isolation of strains B1–12 involved competitive growth of the Hfr strains with strain W1655 F^+ (although growth was limited as far as possible) we should not expect to isolate very defective Hfr strains. However, one class of Hfr types which might still have been isolated was not observed; although at all stages in their isolation strains B1–12 were kept in broth, none had any nutritional requirements additional to those of the ancestral strain. Also, like strain W1655 F^+, they were able to utilize lactose.

Another possible explanation for the experimental result that strains B7, 9 and 10 grow more slowly than strain W1655 F^+ is that growth rate is determined by the length of the chromosome, the DNA of which is replicated at a constant rate, and

that Hfr strains, with the sex factor inserted into the chromosome, grow more slowly than the F+ strains because they have a longer chromosome. However this is excluded on two grounds. In the first place, it predicts that all Hfr strains should grow more slowly than F+ ancestors; this was not observed with strain B1. Secondly, the predicted slowing in growth rate would be of the order of 1–2% (the size of the sex factor relative to that of the chromosome, a difference which is undetectable by the methods used in these experiments). The observed slowing in growth rate for strains B7, 9 and 10 was about 21%.

SUMMARY

The isolation and characterization of an isogenic series of twelve Hfr strains is described. From their points of origin it was concluded that the sex factor integrates at a limited number of sites on the bacterial chromosome. Although the nutritional requirements of these Hfr strains were similar to those of the parent F+ strain, it was observed that one group had a markedly slower growth rate. The relevance of these observations to theories on the nature of F+ fertility and on the formation of Hfr strains is discussed.

REFERENCES

ADELBERG, E. A. & BURNS, S. N. (1960). Genetic variation in the sex factor of *E. coli*. *J. Bact.* **79**, 321–330.

CAMPBELL, A. M. (1962). Episomes. *Adv. Genet.* **11**, 101–145.

CAVALLI-SFORZA, L. L. & JINKS, J. L. (1956). Studies on the genetic system of *E. coli* K-12. *J. Genet.* **54**, 87–112.

CAVALLI-SFORZA, L. L. & LEDERBERG, J. (1955). Isolation of preadaptive mutants in bacteria by sib-selection. *Genetics*, **41**, 367.

CLOWES, R. C. & ROWLEY, D. (1954). Some observations on linkage effects in genetic recombination in *Escherichia coli* K12. *J. gen. Microbiol.* **11**, 250–260.

DEWITT, S. K. & ADELBERG, E. A. (1962). Transduction of the attached sex factor of *Escherichia coli*. *J. Bact.* **83**, 673–678.

HAYES, W. (1953). Observations on a transmissible agent determining sexual differentiation in *Bact. coli*. *J. gen. Microbiol.* **8**, 72–88.

HIROTA, Y. & SNEATH, P. H. A. (1961). F′ and F-mediated transduction in *Escherichia coli* K12. *Jap. J. Genet.* **36**, 307–318.

JACOB, F. & WOLLMAN, E. L. (1956). Recombinasion génétique et mutants de fertilite chez *E. coli* K12. *C. r. hebd. Seanc. Acad. Sci. Paris*, **242**, 303–306.

JACOB, F. & WOLLMAN, E. L. (1961). *Sexuality and the Genetics of Bacteria*. New York and London: Academic Press.

LEDERBERG, J. (1947). Gene recombination and linked segregations in *E. coli*. *Genetics*, **32**, 505–525.

LEDERBERG, E. & LEDERBERG, J. (1953). Genetic studies of lysogenicity in *E. coli*. *Genetics*, **38**, 51–64.

LEDERBERG, J. & TATUM, E. L. (1946). Novel genotypes in mixed cultures of biochemical mutants of bacteria. *Cold Spring Harb. Symp. quant. Biol.* **11**, 113–114.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology*, **1**, 190–206.

LURIA, S. E. & DELBRÜCK, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*, **28**, 491–511.

MÄKELÄ, P. H. (1963). Hfr males in *Salmonella abony*. *Genetics*, **48**, 423–429.

MATNEY, T. S., GOLDSCHMIDT, E. P., ERWIN, N. S. & SCROGGS, R. A. (1964). A preliminary map of genomic sites for F-attachment in *Escherichia coli* K12. *Biochem. biophys. Res. Commun.* **17**, 278.

REEVES, P. (1959). Ph.D. Thesis, London University.

RITCHER, A. (1961). Attachment of wild-type F factor to a specific chromosomal region in a variant strain of *E. coli* K12: the phenomenon of episomic alternation. *Genet. Res.* **2**, 335–345.

SANDERSON, K. E. & DEMEREC, M. (1965). The linkage map of *Salmonella typhimurium*. *Genetics*, **51**, 897–913.

S13 *Hfr Formation and F⁺ Fertility*. P. Broda (Department of Molecular Biology, University of Edinburgh, Kings Buildings, Edinburgh EH9 3JR, Scotland).

Hfr formation and the efficiency of chromosome transfer by an F^+ strain of *Escherichia coli* K12 were reduced to an equal extent (x 100-fold) by the *recA* mutation (1). This suggests that both processes depend upon recombination between homologous regions. Type II strains, which were thought to suffer a defect in Hfr formation (2), apparently do produce fertile clones but have a secondary defect which affects the persistence of these clones. There was a gross asymmetry in the distribution of origins of transfer among over 200 Hfr strains formed by the integration of an $F_{ts}lac^+$ plasmid into the chromosome of a strain carrying a large deletion in the *lac pro* region. This also suggests that not all Hfr clones that arise can persist equally successfully.

1. Cullum, J. and Broda, P. (1979) *Plasmid* 2, 358.
2. Curtiss, R. and Renshaw, J. (1969) *Genetics* 63, 7.

Molec. Gen. Genetics 113, 166–173 (1971)

Isolation and Characterisation of Hfr Strains from a Recombination-Deficient Strain of *Escherichia coli*

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Received July 23, 1971

Summary. A T6^s RecA$^-$ strain carrying a *lac proB* deletion and F_{ts} *lac*$^+$ was challenged with phage T6 and survivors which were both T6^r and Lac$^+$ at 42° were tested for fertility. Among these were a number of Hfr strains which had their points of origin at or near the *tsx* locus and which still carried the *recA* allele. These arose in comparable frequencies in the RecA$^-$ strain and in a Rec$^+$ analogue. We conclude that such integration does not require the RecA function. The rate of chromosome transfer was similar in one such RecA$^-$ Hfr and its Rec$^+$ derivative; the yield of recombinants from the RecA$^-$ strain was slightly lower than from its Rec$^+$ derivative.

Introduction

The formation of Hfr strains in *Escherichia coli* is thought to involve the integration of the sex factor F into the chromosome by a reciprocal recombination event of the Campbell (1962) type between a circular F factor (Freifelder, 1968) and a circular chromosome (Cairns, 1963). It has been shown that F13, which is known to carry markers on both sides of the integrated sex factor (Broda, Beckwith, and Scaife, 1964) is a single circular structure (Freifelder, 1968) confirming that F is integrated into the continuity of bacterial chromosome. Deletions lacking both F and chromosomal functions have now been isolated from Hfr strains (Ippen, Achtman, Willetts, and Fomitchev, in preparation), again indicating that they are continuous as one structure.

Since in F$^+$ *recA* strains the fertility with respect to chromosome transfer has been observed to be about 3% of that in the corresponding F$^+$ *rec*$^+$ strains (Clowes and Moody, 1966), it is possible that Hfr formation is a *recA*-dependent function. However, since *recA* strains are poor donors *per se*, as measured by the transfer of an F-prime factor (see Willetts and Broda, 1969, for discussion of this point), and since it is not known what proportion of F fertility is in fact due to stable Hfr clones (Broda, 1967; Curtiss and Stallions, 1969), it is also possible that Hfr formation is *recA*-independent, as are some other recombinational processes (Franklin, 1967; Inselburg, 1967). In the present communication we report the use of a selective technique to isolate Hfr strains from a *recA* parent, and describe some of their properties.

Material and Methods

Bacterial Strains. The strains used are listed in Table 1. ED2444 is an *spc* derivative of strain W3110 (*trpC his*). It carries the *lac pro* deletion X111 (Signer and Beckwith, 1966) the *recA56* allele (Clark, 1967) and the temperature-sensitive F_{ts114} *lac* (Cuzin and Jacob, 1967).

Phage Strains. Phages *λc* and *λfec* (= *λpbio* C^+_{III}; Manly *et al.*, 1969) were provided by Dr. M. Monk, phage MS2 by Dr. N. Willetts and phage T6 by Dr. M. Achtman.

Media. These have been described by Willetts and Finnegan (1970).

Table 1. *Bacterial strains used*[a]

train	Genotype	Origin
D2444	△ *lac pro*$_{X111}$ *his tsx*$^+$ *trp spc recA56*/F$_{ts114}$ *lac*$^+$	See text
D2446	△ *lac pro*$_{X111}$ *his tsx*$^+$ *trp spc rec*$^+$/F$_{ts114}$ *lac*$^+$	See text
D2410	F$^-$△ *lac pro*$_{X111}$ *his tsx*$^+$ *trp spc recA56*	See text
D2409	F$^-$△ *lac pro*$_{X111}$ *his tsx*$^+$ *trp spc rec*$^+$	See text
B257	Hfr Cavalli *met* (*λ*)$^+$	N. S. Willetts
C411	F$^-$ *leu his argG metB str*	N. S. Willetts
X149	F$^-$ *leu his argG metB str recA*	JC411
B314	F$^-$ *lac purE tsx str*	1177 (Broda *et al.*, 1964)
D1111	F$^-$ *lac purE tsx str recA*	PB314
C5462	F$^-$ *his spc str lac*$_{\Delta X74}$	N. S. Willetts
C5029	Hfr KL16 *thr ilv spc*	N. S. Willetts
C5088	Hfr KL16 *thr ilv spc recA56*	N. S. Willetts
T713	F$^-$ *lysA argA cysC str mal xyl mtl thi*	N. S. Willetts

[a]The nomenclature used is that recommended by Demerec *et al.* (1966) and Taylor (1970).

Isolation of Hfr Strains. A 5 ml standing overnight culture of the strain ED2444, grown 30°, was centrifuged and resuspended in the same volume of fresh broth. After incubation 42° for 30 minutes, phage T6 was added at a final concentration of 5×10^{10} phage particles r ml, and the mixture was incubated further at 42° for another 30 minutes. It was then ntrifuged, the bacterial pellet was resuspended in 0.1 volume of L broth, and 0.1 ml aliquots ere plated on EMB lactose plates. These were incubated at 42° for 48 hours. About 150 colo- es were recovered per plate; about half of these were Lac$^+$. These were screened in patch sts for clones which were presumptive Rec$^-$ Hfr clones (see Results).

Mating Conditions. Matings were at 37° between exponential broth cultures, at about $\times10^7$ males per ml and 2×10^8 females per ml, unless otherwise stated. Aliquots of 0.1 ml ere plated in 2.5 ml soft agar containing 0.1 ml broth. Hfr strains to be used as recipients ere converted to the F$^-$ phenocopy state by shaking at 37° for 20 hours. A 0.5 ml aliquot was oculated into 1 ml of fresh broth, and 0.5 ml of the exponentially growing donor Hfr strain, 2×10^8 cells per ml, was then added. In interrupted matings mating pairs were separated ing the method of Low and Wood (1965). Frequencies of recombinant formation are expressed recombinants/ml/Klett unit of donor bacteria because of the low and somewhat variable able counts obtained in platings from cultures of RecA$^-$ bacteria.

Phage Plating. Phage T6 was plated as a 0.1 ml aliquot (2×10^3 plaque forming units r ml) together with 0.3 ml of a late exponential bacterial culture (4×10^8 cells/ml) and 5 ml of LC top agar, on L nutrient plates. Phage MS2 was plated with 5×10^{-3} *M* Ca^{++} in top agar, on L nutrient plates, and phages *λc* and *λfec* were plated on Difco nutrient ates with bacteria which had been prestarved in 10^{-2} *M* $MgSO_4$ for 60 minutes at 37°.

Acridine Orange Curing. 0.05 ml of bacteria containing about 5×10^3 cells were inoculated to 2 mls of L broth at pH 7.8, together with 20, 25, and 50 μg/ml of acridine orange, and cubated in the dark overnight.

Ultraviolet Light Sensitivity. Exponential cultures at about 2×10^8 cells/ml were diluted 0-fold in buffer. 5 mls were irradiated, and aliquots were plated on L nutrient plates, which ere incubated at 37° in the dark for 48 hours.

Results

Isolation of Donor Strains

The rationale behind the isolation of the strains described below was to obtain om a T6^s strain carrying F$_{ts}$ *lac*$^+$ derivatives which had simultaneously acquired e ability to utilise lactose at 42° and become resistant to phage T6. It was hoped at some of these would be strains which had become Hfr by integration of the

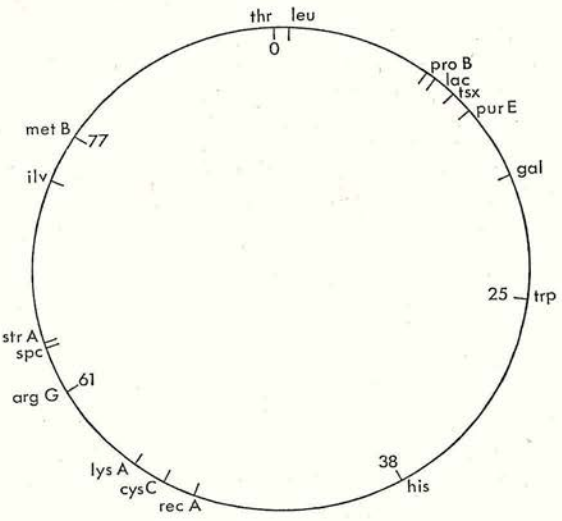


Fig. 1. The genetic linkage map of *E. coli*. *Lac* and *proB* are deleted in strain ED2444 and its analogues. (After Taylor, 1970)

F-prime factor into the *tsx* gene. The isolation of these strains is described in the Materials and Methods section. The criteria used in screening patched clones of the purified presumptive Hfr strains were (a) resistance to phage T6 (b) sensitivity to UV light (c) the ability to utilise lactose on EMB lactose plates at 42° (d) the ability to transfer *leu*$^+$ at higher efficiency than *argG*$^+$ to the F$^-$ strain JC411 (see Fig. 1) and (e) the inability to transfer *lac*$^+$ with high efficiency to a recombination-deficient F$^-$ strain, ED1111. The object of the last of these tests was to confirm that the strains did not carry an autonomous F*lac*$^+$ factor. Of 150 clones screened, the majority fulfilled these criteria and eight were selected for further study. It was noted that the strain ED2446, the *rec*$^+$ analogue of ED2444, gave a similar yield of Lac$^+$ T6^r clones, and of these too the majority fulfilled these criteria.

The eight derivatives of strain ED2444 that were investigated, like their parent strain, all required proline, tryptophan and histidine. In addition, strain ED2482 required a casamino acid supplement for growth at 37°; this strain also grew most slowly in broth culture. All strains, unlike ED2444, were stably Lac$^+$ at 42°. Moreover, under conditions when the parental strain ED2444 was partially cured of the Lac$^+$ phenotype both in the presence and in the absence of acridine orange, all the progeny of these strains remained Lac$^+$.

Transfer Properties

One hour interrupted matings in broth were performed with the eight donor strains. Each strain was mated with JC411, selecting *leu*$^+$, *metB*$^+$ and *argG*$^+$ recombinants, and with PB314 selecting *purE*$^+$ and *lac*$^+$ recombinants. They were also mated with MX149, the *recA* analogue of JC411, for *leu*$^+$ progeny, and with ED1111, the *recA* analogue of PB314, for *purE*$^+$ and *lac*$^+$ progeny. It was found that the eight strains were similar with respect to transfer; the data for crosses

Table 2. *Efficiency of transfer of markers from ED2444 and its derivatives*[a]

Strain	× JC411			× PB314		× MX149	× ED1111	
	Leu⁺	Met⁺	ArgG⁺	PurE⁺	Lac⁺(b)	Leu⁺(b)	PurE⁺	Lac⁺(b)
ED2444	0.0007	<0.0002	<0.0002	<0.0002	15	<0.0002	<0.0002	5.0
ED2446	0.004	0.0002	0.0002	0.0017	36	<0.0002	<0.0002	11
ED2475	1.43	0.003	0.0002	0.0002	0.28	<0.0002	<0.0002	0.062
ED2476	3.5	0.003	0.0003	<0.0002	0.013	<0.0002	<0.0002	0.001
ED2461	1.26	<0.0002	<0.0002	0.0004	0.052	<0.0002	<0.0002	0.016
AB257	7.2	—	0.0015	1.9	15	0.002	0.0002	0.0016

[a] Progeny (× 10^5)/ml/Klett of donor cells.
[b] Incubated at 30°.

Table 3. *The efficiency of plating of various phages upon ED2444 and its analogues*

	Phage			
Strain	T6	MS2	λc	λ*fec*
ED2444	71	56[a]	70	<0.01
ED2446	100	63[a]	100	100
ED2475	$<10^{-6}$	35	59	<0.01
ED2476	$<10^{-6}$	61	72	<0.01
ED2461	$<10^{-6}$	63	104	95
AB257	—	100	—	—
JC411	95	$<10^{-6}$	—	—
PB314	$<10^{-6}$	—	—	—

[a] at 32°.

with two of the strains, ED2475 and ED2476, are presented in Table 2, together with those from control crosses with strains ED2444, ED2446, ED2461 (a *rec*⁺ derivative of ED2476 obtained by mating the latter strain with the *recA*⁺ Hfr JC5029) and AB257 (Hfr Cavalli). These data show that unlike the parent strain ED2444, strains ED2475 and ED2476 transferred *leu*⁺ with high efficiency. *purE* was not transferred with high efficiency. It appears, therefore, that strains ED2475 and ED2476 are indeed Hfr strains transferring *leu*⁺ early and *purE*⁺ late, as would be expected if the F_{ts} *lac*⁺ F-prime factor was integrated in the *tsx* gene. Table 3 shows that these strains are MS2ˢ and resistant to phage T6.

The absence of Leu⁺ progeny in the crosses between ED2475 and ED2476 and MX149 shows that *leu*⁺ transfer is not due to the presence of an F-prime factor carrying *leu*⁺. ED2475 and ED2476 both yield Lac⁺ progeny with both strains PB314 and ED1111. These arise at widely different (but reproducible) frequencies characteristic of the donor strains. We have shown in patch matings that virtually all the Lac⁺ progeny obtained in the crosses using PB314 as the recipient can retransfer the Lac⁺ phenotype to a further Lac⁻ recipient strain, JC5462. We conclude that these are *F'lac*⁺ revertants, and that in both donor strains ED2475 and ED2476 *lac*⁺ is probably transferred terminally on the chromosome.

Recombination Deficiency of the Donor Strains

Strain ED2444 had been isolated as a *recA* derivative, the *recA56* allele coming from strain JC5088. In the initial screening of its derivatives it was confirmed that strains ED2475 and ED2476 had retained the UV-sensitive phenotype; this observation was corroborated by measurements of their survival after UV-irradiation (Table 4). To demonstrate that they were not merely UV-sensitive, but also recombination deficient, they were used as recipients in a mating against the Hfr strains JC5088 (*recA*) and JC5029 (*rec*$^+$), which are otherwise isogenic, and transfer the *recA*$^+$ and *his*$^+$ alleles early. The results are presented in Table 5. Only the *RecA*$^+$ recipients ED2409 and ED2461 gave *his*$^+$ recombinants with JC5088, but all strains gave recombinants with JC5029. We conclude that the *recA* allele is present in the recipient strains (apart from ED2409 and ED2461), and that only when the *recA*$^+$ allele is present or when it is introduced by strain JC5029 can recombinants be formed.

It has been reported (Manly *et al.*, 1969) that a derivative of phage λ, λfec, is able to plate on *recA*$^+$ strains but not on *recA* strains. The conclusion that strains ED2475 and ED2476 are *recA* is confirmed by the observation (Table 3) that λc but not λfec is able to form plaques on them.

Table 4. % *survival of ED2444 and its analogues after ultraviolet irradiation*

Strain	UV dose in ergs/mm^2		
	0	10.75	107.5
ED2444	100	0.01	<0.005
ED2446	100	57	31
ED2475	100	0.03	<0.005
ED2476	100	0.08	<0.01
ED2461	100	73	45

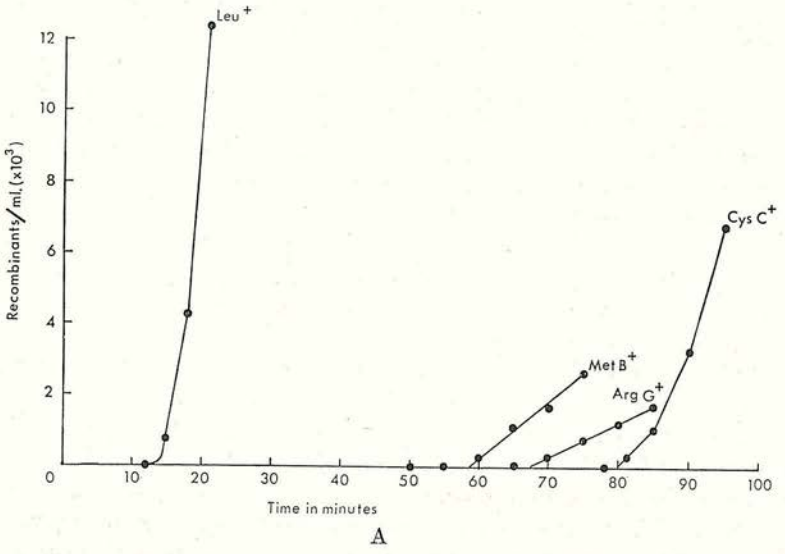
Table 5. *Efficiency of formation of His*$^+$ *recombinants of strain ED2475 and ED2476 and their analogues, using strains JC5029 and JC5088 as donor strains*

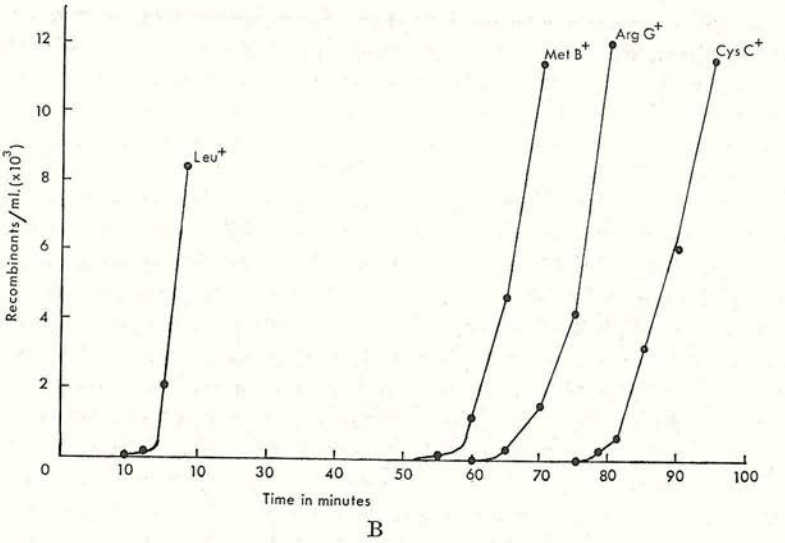
Strain	His$^+$ recombinants ($\times 10^4$)	
	$\times$ JC5029	$\times$ JC5088
ED2475	7.3	<0.0001
ED2476	6.4	<0.0001
ED2461	10	0.67
ED2409	8.4	0.67
ED2410	2.0	<0.0001

Expressed as His$^+$/ml/Klett of donor bacteria.

Interrupted Mating Experiments

Strain ED2476 was compared with its *rec*$^+$ derivative strain ED2461 for the rate of chromosome transfer in interrupted mating experiments. *leu*$^+$, *argG*$^+$ and *metB*$^+$ recombinants were obtained in crosses with JC411, and AT713 was used to obtained *cysC*$^+$ recombinants. Time of entry curves are presented in Fig. 2A





ig. 2A and B. Kinetics of chromosome transfer by ED2476 and ED2461 in crosses with JC411 (for Leu⁺, MetB⁺ and ArgG⁺ recombinants) and AT713 (for CysC⁺ recombinants)

ED2476) and 2B (ED2461); the inferred times of entry of the different markers s well as the absolute yields of each class of recombinants are given in Table 6. can be seen that the two Hfr strains are very similar with respect to the time f entry of markers. The absolute yields of each class of recombinant from the ross with ED2476 are lower than those from the crosses with ED2461. The ratio f these yields decreases from 0.7 for *leu*⁺ to 0.3 for *cysC*⁺.

An unexpected finding was that the *metB*⁺ allele appeared to be much closer to *rgG*⁺ and *cysC*⁺ and further from *leu*⁺ than was expected on the basis of the

Table 6. *Transfer of markers from strains ED2476, ED2461, and ED2468*

Marker selected	Map position[a]	ED2476		ED2461		Ratio of recombinant yields	ED2468
		Time of entry	Yield of recombinants[b]	Time of entry	Yield of recombinants[b]		Time of entry
Leu$^+$ (× JC411)	1	14	3.0	14	4.1	0.7	20
MetB$^+$ (× JC411)	77	58	0.20	58	0.41	0.5	35
ArgG$^+$ (× JC411)	61	67	0.13	63	0.30	0.4	65
CysC$^+$ (× AT713)	53	80	0.11	77	0.42	0.3	84

[a] After Taylor (1970).
[b] 2 hr matings. Recombinants expressed as recombinants (× 10^5)/ml/Klett of donor cells.

chromosome map of Taylor (1970). In a control experiment, therefore, the times of entry of the *leu*$^+$, *metB*$^+$, *argG*$^+$ and *cysC*$^+$ alleles were determined in crosses in which ED2468, a *metB*$^+$ derivative of AB257, was used as the donor. The times of entry inferred from these crosses are also presented in Table 6. It will be noted that although *argG*$^+$ and *cysC*$^+$ are transferred at similar times as by ED2476 and ED2461, *metB*$^+$ is now transferred at about the expected time. We conclude that the delayed transfer of *metB*$^+$ by ED2476 and ED2461 is a pecularity of these donor strains; it could be due to a transposition of the *metB*$^+$ locus.

Discussion

It has been shown previously that the integration of an F-prime factor into a chromosome with which it has homology occurs very much more frequently in a Rec$^+$ strain than in a RecA$^-$ strain (Bastarrachea and Clark, 1968; Wilkins, 1969; DeVries and Maas, 1971; Nishimura *et al.*, 1971). We have shown that Hfr strains can arise by the integration of an F_{ts} *lac* F-prime factor into a *recA* strain carrying a large *lac pro* deletion, so that no apparent homology exists between the F-prime factor and the chromosome. By a number of criteria such strains are both Hfr and *recA*, and it seems likely that these have arisen by the integration of the F_{ts} *lac* into the *tsx* locus. We calculate that the rate at which this occurs is of the order of 10^{-7} per T6^s F_{ts} *lac* cell, and that this rate is comparable to that in the *rec*$^+$ analogue. We conclude that this infrequent integration involving interaction of apparently non-homologous regions of DNA (the *tsx* gene and the F_{ts} *lac* particle) occurs by a process independent of the *recA* function, by which it would normally be obscured. It is possible that it is another in the category of "extraordinary recombination events" characterised by independence of the *recA* function, and described by Franklin (1967) and Inselburg (1967).

When one *recA* isolate, ED2476, was compared with its *rec*$^+$ derivative, strain ED2461, it was found that the rates of chromosome transfer were identical. However, the absolute yield of recombinants (as measured/ml/Klett unit of donor cells) was slightly lower in the *recA* strain; this difference became more marked the further from the origin the marker was situated. The conclusion that the *recA* function has no gross effect on chromosome transfer complements the observation of Greenberg *et al.* (1970) that transfer by a RecA$^-$ Hfr strain was no more sensitive to UV-irradiation than transfer by a Rec$^+$ strain.

References

Bastarrachea, F., Clark, A. J.: Isolation and characterisation of an *Escherichia coli* strain harbouring three sex factors. Genetics **60**, 641–660 (1968).

Broda, P.: The formation of Hfr strains in *Escherichia coli* K12. Genet. Res. **9**, 35–47 (1967).

– Beckwith, J. R., Scaife, J.: The characterisation of a new type of F-prime factor in *Escherichia coli* K12. Genet. Res. **5**, 489–494 (1964).

Cairns, J.: The bacterial chromosome and its manner of replication as seen by autoradiography. J. molec. Biol. **6**, 208–213 (1963).

Campbell, A. M.: Episomes. Advanc. Genet. **11**, 101–145 (1962).

Clark, A. J.: The beginning of a genetic analysis of recombination proficiency. J. cell. Physiol. **70**, Suppl. 1, 165–180 (1967).

Clowes, R. C., Moody, E. E. M.: Chromosome transfer from recombination-deficient strains of *Escherichia coli* K-12. Genetics **53**, 717–726 (1966).

Curtiss III, R., Stallions, D. R.: Probability of F integration and frequency of stable Hfr donors in F⁺ populations of *Escherichia coli* K-12. Genetics **63**, 27–38 (1969).

Cuzin, F., Jacob, F.: Mutations de l'épisome F d'*Escherichia coli* K12. II. Mutations à replication thermosensible. Ann. Inst. Pasteur **112**, 397–418 (1967).

Demerec, M., Adelberg, E. A., Clark, A. J., Hartman, P. E.: A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**, 61–76 (1966).

DeVries, J. K., Maas, W. K.: Chromosomal integration of F′ factors in recombination-deficient Hfr strains of *Escherichia coli*. J. Bact. **106**, 150–156 (1971).

Franklin, N. C.: Extraordinary recombinational events in *Escherichia coli*. Their independence of the *rec*⁺ function. Genetics **55**, 699–707 (1967).

Freifelder, D.: Studies on *Escherichia coli* sex factors. III. Covalently closed F′*lac* DNA molecules. J. molec. Biol. **34**, 31–38 (1968).

Greenberg, J., Green, M. H. L., Bar-Nun, N.: The effect of UV irradiation on the capacity of an Hfr RecA strain of *Escherichia coli* to act as donor. Molec. gen. Genet. **107**, 209–214 (1970).

Inselburg, J.: Formation of deletion mutants in recombination-deficient mutants of *Escherichia coli*. J. Bact. **94**, 1266–1267 (1967).

Low, B., Wood, J. H.: A quick and efficient method for interruption of Bacterial Conjugation. Genet. Res. **6**, 300–303 (1965).

Manly, K. F., Signer, E. R., Radding, C. M.: Non-essential functions of bacteriophage λ. Virology **37**, 177–188 (1969).

Nishimura, Y., Caro, L., Berg, C. M., Hirota, Y: Chromosome replication in *Escherichia coli*. IV. Control of chromosome replication and cell division by an integrated episome. J. molec. Biol. **55**, 441–456 (1971).

Signer, E. R., Beckwith, J. R.: Transposition of the *Lac* region of *Escherichia coli*. III. The mechanism of attachment of bacteriophage ø80 to the bacterial chromosome. J. molec. Biol. **22**, 33–51 (1966).

Taylor, A. L.: Current linkage map of *Escherichia coli* K12. Bact. Rev. **34**, 155–175 (1970).

Wilkins, B. M.: Chromosome transfer from F*lac*⁺ strains of *Escherichia coli* K12 mutant at *recA*, *recB* or *recC*. J. Bact. **98**, 599–604 (1969).

Willetts, N., Broda, P.: The *Escherichia coli* sex factor. *in* Ciba Foundation Symposium *on* Bacterial Episomes and Plasmids, edit. by G. E. W. Wolstenholme and Maeve O'Connor, p. 32–48. London: J. and A. Churchill 1969.

– Finnegan, D.: Characterisation of *E. coli* K12 strains carrying both an F prime and an R factor. Genet. Res. **16**, 113–122 (1970).

Communicated by R. Pritchard

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PLASMID 2, 358–365 (1979)

Chromosome Transfer and Hfr Formation by F in *rec*$^+$ and *recA* Strains of *Escherichia coli* K12

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Received April 17, 1978

We attempted to assess the role of Hfr clones in chromosome transfer by F$^+$ populations. We thought that any Hfr-independent component of fertility might be affected to a different extent by the *recA* mutation than was the Hfr component. However, the rate of Hfr formation and the efficiency of chromosome transfer were reduced to an equal extent (×100-fold) by the *recA* mutation. Such experiments therefore provide no evidence for an Hfr-independent component. It appeared that Type II strains, which were thought to suffer a defect in Hfr formation, actually produced fertile clones but had a secondary defect which affected the persistence of these clones. Thus, evidence from Type II strains is also not useful for examining the quantitative contribution of Hfr cells to F$^+$ transfer.

The question of whether all chromosome transfer from F$^+$ strains of *Escherichia coli* can be accounted for by transfer from Hfr clones within the population (as suggested by Jacob and Wollman, 1956) is still unresolved. Three studies have suggested that some other process may be responsible for much of the transfer. Reeves (1960) found that most recombinants in F$^+$ × F$^-$ matings were F$^+$, whereas pure Hfr strains gave predominantly F$^-$ recombinants. Broda (1967) showed that the distribution of nonselected markers in F$^+$ × F$^-$ matings could not be accounted for by the pattern of Hfr strains that he was able to isolate from the F$^+$ strain. Curtiss and Renshaw (1969a) discovered strains, which they called Type II strains, that seemed defective in Hfr formation although they transferred chromosomal markers with a similar efficiency to normal (Type I) strains. This was a property of the cell itself rather than one conferred by the particular F factor. Whatever the precise nature of the donor cells, physical interaction between F and the chromosome is likely, since the efficiency of transfer of chromosomal genes was reduced about 30-fold by the *recA* mutation (Clowes and Moody, 1966). This contrasts with the mobilisation of *colE1* by the F transfer system (Alfaro and Willetts, 1972).

Hfr formation requires reciprocal recombination (Campbell, 1962) between homologous DNA segments, one on F and the other on the chromosome (Ohtsubo *et al.*, 1974; Hu *et al.*, 1975; Deonier and Davidson, 1976). Such integration could well be *recA*-dependent, as F excision appears to be (Deonier and Mirels, 1977). However, since at least two such regions appear to be identical to "insertion sequences" which can be inserted and excised at nonhomologous regions in *recA* strains (for a review, see Starlinger and Saedler, 1976), it could also be *recA* independent. Hfr formation from F′ factors in *recA* strains has already been described (Broda and Meacock, 1971; DeVries and Maas, 1971). However, the frequency of such events is difficult to assess, and Hfr formation in *recA* F$^+$ strains has not been reported.

If, as suggested above, transfer in F$^+$ populations is partially independent of Hfr clones, the two processes (Hfr-dependent and Hfr-independent fertility) would be resolved by any differential effect upon them of the *recA* allele. This was tested in two ways. First, the efficiencies of chromosome

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transfer by Type I and Type II (non-Hfr forming) *recA* F^+ strains were compared. If only Hfr formation were *recA* independent, chromosome transfer from a Type II *recA* strain should be relatively very inefficient. This proved not to be the case. Second, the effects of the *recA* mutation on the overall fertility and on the rate of Hfr formation in normal (i.e., Type I) strains were determined. We found that they are depressed about equally. Analogous experiments with the Type II rec^+ and *recA* strains suggested that the defect in Type II strains was secondary rather than a defect in Hfr formation itself.

MATERIALS AND METHODS

Media. The minimal media and L-broth were described by Cullum *et al.* (1978). L-broth was used for all liquid cultures and for dilutions. Giemsa agar was (per litre) 10 g tryptone (Difco), 10 g yeast extract (Difco), 15 g agar (Difco), 8 g NaCl, and 1 litre distilled water. This agar was melted and 2 mM $CaCl_2$, 1% glucose, and 12.5 ml/litre of Giemsa (Gurr R66) were added.

Bacterial and phage strains. The bacterial strains are shown in Table 1. High-titre phage T6 was prepared as in Cullum *et al.* (1978). Plate lysates of the male specific phage MS2 were prepared by growth on strain ED879.

The Type I F^+ strains were derived from χ637 and the Type II F^+ strains were derived from χ852. Thy^- derivatives of the parent strains were selected with trimethoprim (10 μg/ml). These were mated with the *recA* Hfr strain JC5088 and *recA* and rec^+ clones were chosen from among the Thy^+ progeny. These were made F^+ by mating with ED17, which carries the F factor transferred from the original $K12(\lambda)^+F^+$.

Fluctuation test. A standing overnight culture was diluted to about 200 cells/ml and 0.5-ml samples were dispensed into a series of small tubes (12 × 75 mm). A parallel bulk culture (volume at least 50 ml) was

TABLE 1

BACTERIAL STRAINS

Strain	Genotype	Derivation
ED17	F^+ *his trp lys str tsx gal lac*	N. Willetts
χ637	F^- *lacY cyc* $(\lambda)^-$	R. Curtiss; type I strain derived from W1485. Curtiss and Renshaw (1969a)
χ852	F^- *lac cyc thi* $(\lambda)^-$	R. Curtiss; type II strain derived from W945. Curtiss and Renshaw (1969a)
JC5088	Hfr *spc thr leu ilv thi rel recA56* $(\lambda)^-$	A. J. Clark; A KL16 derivative. See Bachmann (1972)
χ478	F^- *ara leu ton proC lacZ tsx purE trp lys str xyl mtl metE thi*	R. Curtiss; Curtiss and Renshaw (1969a)
AB1157	F^- *thr leu thi lacY galK ara xyl mtl proA his argE str tsx sup37*	See Bachmann (1972)
ED877	F^+ *lacY cyc recA56* $(\lambda)^-$	From χ637 (this paper)
ED879	F^+ *lacY cyc* $(\lambda)^-$	From χ637 (this paper)
ED957	F^+ *lac cyc thi* $(\lambda)^-$	From χ852 (this paper)
ED969	F^+ *lac cyc thi recA56* $(\lambda)^-$	From χ852 (this paper)
AB259	HfrH *thi rel* $(\lambda)^-$	See Bachmann (1972)

also prepared from the same dilution. These cultures were incubated until they contained about 2×10^8 cells/ml. Then 0.1-ml samples were dispensed into small tubes (the samples were previously diluted 1 in 20 for *rec*$^+$ strains), and the parent cultures were then stored at 4°C. The samples were incubated for 5 min and then 0.1 ml per tube of an exponentially growing culture of AB1157 was added. After 80 min mating, phage T6 was added to a final titre of about 10^{10}/ml. After 5 min further incubation, the total contents of each tube was plated on agar selective for Thr$^+$Ara$^+$Leu$^+$Str^R recombinants using top agar. It was essential to select for all three nutritional markers to reduce the effects of background growth and mutations.

Streak matings. Cultures (0.5 ml) were grown as described for the fluctuation tests. Loopfuls (0.003 ml) of the cultures were streaked on agar selective for Thr$^+$Ara$^+$Leu$^+$Str^R recombinants after each plate had been spread with 0.5 ml of an exponentially growing culture of AB1157 concentrated 10-fold by centrifugation. Twenty-five streaks per plate were used.

Sib selection and isolation of Hfrs. The method of sib selection, as used previously (Broda 1967), was used to help isolate Hfr clones. Portions (0.5 ml) of a suitable dilution of a culture were dispensed into small tubes and after incubation were mated with AB1157 in small tubes, as described for the fluctuation tests. When the number of recombinants was sufficiently high, clones were tested for the Hfr property by replica plating onto agar selective for Thr$^+$Ara$^+$Leu$^+$Str^R progeny which had been spread with 0.1 ml of an exponentially growing culture of AB1157. Otherwise, the most fertile culture was used for another cycle of sib selection.

Testing for MS2 sensitivity. Colonies were patched onto L-broth agar using sterile toothpicks. After a few hours growth they were replica plated onto Giemsa plates spread with 0.1 ml of phage MS2 (about 3×10^{12} pfu/ml). After overnight incubation, the MS2^S (that is, F$^+$) clones gave purple-coloured patches that could be distinguished from the white patches given by F$^-$ clones.

Origin and direction of transfer of Hfr strains. Hfr strains were mated with χ478 in a ratio of 1:100. Samples were taken at 20, 40, and 60 min and dilutions were plated on agar selective for Pur$^+$, Met$^+$, Leu$^+$, Pro$^+$, and Trp$^+$ recombinants that were streptomycin resistant.

Matings to determine the efficiency of transfer of chromosomal genes. Cultures of the parent F$^+$ strains and AB1157 were grown to $1-2 \times 10^8$ cells/ml. A 2.5-ml sample of the donor culture was added to 50 ml of the AB1157 culture. After 60 min mating phage T6 was added to a final concentration of about 10^{10} pfu/ml to kill the donors. After 5–10 min the cultures were centrifuged and the cells resuspended in 0.02–0.1 volume of L-broth. Dilutions were plated on agar selective for the Thr$^+$Ara$^+$Leu$^+$Str^R progeny and agar selective for the AB1157 recipients.

When the progeny were to be tested for MS2 sensitivity, the matings were done with a 1:30 donor-to-recipient ratio, and control matings using F$^+$ cultures to which 1% Hfr had been added were done under the same conditions. The Hfr strains used for this purpose were the *rec*$^+$ strain AB259 and one of the *recA* Hfr strains that we had isolated from strain ED877. Twenty-five of the progeny colonies from each mating were tested for MS2 sensitivity as described above.

Estimation of rates of Hfr formation. We used the results of fluctuation tests to calculate the rate of Hfr formation per cell per generation. The experiments were designed to detect those cells that transfer the *thr ara leu* region efficiently. We therefore have assumed that Hfr formation and/or chromosome transfer from this region are representative of these processes for the whole chromosome. We have also assumed that Hfr cells have the same growth rate as F$^+$ cells. The possibility that two processes contribute to chromosome transfer by F$^+$

strains means that the methods of Luria and Delbrück (1943) cannot be used to estimate the rate of Hfr formation. However, as the non-Hfr fertility would have only a small variance between cultures, the rate of Hfr formation can be estimated from the number of "jackpot" cultures. The method is illustrated using values we obtained for the Type I *rec*$^+$ strain ED879 (see Results).

Cultures which give rise to a large number of progeny should have a significant contribution from Hfr cells. We chose a cutoff value (in this case 59 progeny per mating tube) such that the "fertile" tubes with more than this number of progeny are likely to contain a large clone of Hfr cells. The probability of a single large clone will be much higher than that of two clones of half the size arising independently in the same culture. The difference between the number of progeny in a fertile tube and the mean number of progeny (ignoring "jackpot" tubes) is assumed to be due to the Hfr clone. Thus, in our case the mean number of progeny (ignoring the "jackpot" cultures) is 36.4, so that the fertile tubes have more than 22.6 (=59 − 36.4) progeny due to the Hfr clone. As the mating tube contained 0.1 ml of a 1/20 dilution of the donor culture and the efficiency of mating is assumed to be 0.1, this corresponds to more than $22.6 \times 10 \times 20 \times 10$ Hfr cells/ml in the culture, or (as the culture volume is 0.5 ml) 22.6×10^3 Hfr cells/culture. The culture contained 4.1×10^8 cells/ml (Table 3) or 2.05×10^8 cells/culture. Therefore, the Hfr clone arose when there were no more than $2.05 \times 10^8/22.6 \times 10^3 = 9 \times 10^3$ cells in the culture, assuming Hfr and F$^+$ growth rates are equal. This means that at most twice this number, i.e., 1.8×10^4, of cell generations had passed in the fertile cultures before the Hfr was formed. If α is the probability that an Hfr is formed in a single-cell generation (i.e., α is the Hfr formation rate), then the probability that an Hfr is not formed is $1 - \alpha$. Thus, the probability that no Hfr is formed in 1.8×10^4 cell generations, i.e., the culture is not a "fertile culture," is given by $(1 - \alpha)^{1.8 \times 10^4}$. We estimate this probability as 0.94, because in 47 out of 50 cultures no such Hfr is formed: so we obtain $(1 - \alpha)^{1.8 \times 10^4} = 0.94$. From this we can calculate that α has the value 3.4×10^{-6} per cell per generation.

RESULTS

Efficiency of Transfer of Chromosomal Genes

Table 2 shows the results of an experiment comparing the ability of Type I and Type II *rec*$^+$ and *recA* strains to transfer chromosomal genes. This showed that the efficiencies of transfer by the Type I and Type II strains were very similar. However, the *recA* strains transfer about 100-fold less efficiently than their *rec*$^+$ sister strains. This is not due to a general reduction in mating efficiency as F transfer occurs at a comparable frequency in the *rec*$^+$ and *recA* strains.

If the proportion of chromosome transfer that is due to Hfr cells were different in the four donor strains, the proportion of chromosomal recombinants which were F$^-$ might be expected to vary, as Hfr strains produce predominantly F$^-$ progeny. We therefore performed matings (data not shown) to measure the proportion of F$^-$ progeny. In all four cases the proportion of F$^-$ chromosomal recombinants was about 20%. Control matings were done with a small proportion of Hfr cells added to the F$^+$ cultures (as in Reeves, 1960), so that most of the chromosomal recombinants (more than 99%) were due to the Hfr donors. About one-third of the recombinants were F$^+$, presumably mainly due to multiple mating of recipient cells with both Hfr and F$^+$ donors. Thus, in our mating conditions, multiple mating reduces the number of F$^-$ progeny by one-third. This suggests that about 30% (rather than 20%) of the chromosomal recombinants in our F$^+$ × F$^-$ matings would be F$^-$ if there were no multiple matings. The similar proportion of F$^-$ recombinants in the *recA* and *rec*$^+$ cases suggests that Hfr

TABLE 2

EFFICIENCY OF TRANSFER BY DONOR STRAINS

Donor	ED879	ED877	ED957	ED969
Donor type	I	I	II	II
recA allele	+	−	+	−
Recipient viable count at start of mating ($\times 10^{-8}$/ml)	1.3	2.0	1.3	1.6
Initial ratio of donors to recipients (x)	0.15	0.063	0.12	0.059
Final ratio of Str^R Thr^+ Ara^+ Leu^+ progeny to recipients (y) ($\times 10^9$)	690	2.6	480	4.0
Efficiency of mating (y/x) ($\times 10^8$)	460	4.2	400	6.7
Proportion of recipient cells which become F^+	0.18	0.18	0.14	0.12

formation is depressed in *recA* strains to a similar extent to overall fertility.

Type I rec⁺ Strain

In a fluctuation test for fertility, as devised by Jacob and Wollman (1956), a series of parallel cultures of an F^+ strain are grown from small inocula (about 100 cells). If Hfr formation occurs, the variance in the number of progeny between cultures will be large because of high-fertility cultures ("jackpots") in which an Hfr clone has arisen early by chance.

Table 3, line 1 shows the results of such an experiment using ED879. Much of the variance was due to the three most fertile cultures (1430, 128, and 78 progeny). Line 2 shows a control experiment where all the samples for mating were taken from the same bulk culture so that there was little variation in fertility.

We isolated Hfr strains from the three cultures mentioned above, using a sib selection method (Broda, 1967). Matings with χ478 showed a gradient of transfer, with the origin of transfer between *leu* (an early marker) and *proC* in all three cases.

We used the results of this fluctuation test to estimate the rate of Hfr formation. Reconstruction experiments showed that the efficiency of mating of the Hfr strains that we isolated was about 0.1 progeny per donor. We used a cutoff value of 59 progeny (this was three standard deviations above the mean when the three most fertile cultures were ignored). No other cultures gave that many progeny. These data allowed us to calculate a rate of Hfr formation of

TABLE 3

FLUCTUATION TESTS FOR THE *rec⁺* STRAINS[a]

Donor strain	Number of matings	Donor viable count ($\times 10^{-8}$/ml)	Mean number of progeny per plate ($\bar{x}$)	Variance in number of progeny (s^2)	$s^2/\bar{x}$
1. ED879 (Type I)	50	4.1	67.0	38900	582
2. Bulk culture control of ED879	50	3.9	26.2	52.6	2.01
3. ED957 (Type II)	50	4.4	20.4	57.5	2.81
4. Bulk culture control for ED957	50	4.6	19.7	22.5	1.14
5. ED957 (Type II)	98	3.8	14.8	857	57.9

[a] Tests 1 and 3 were performed in parallel with tests 2 and 4, respectively.

3.4×10^{-6}/cell/generation (see Materials and Methods).

Type I recA Strain

Neither of the two fluctuation tests with the Type I *recA* strain ED877, using 50 cultures and 100 cultures, respectively, gave significant variation in fertility and no highly fertile cultures were observed (data not shown). Thus, the Hfr formation rate appears lower in the *recA* strain than in the *rec*$^+$ strain.

We then used a streak mating technique to screen a larger number of cultures. We found one highly fertile culture in 449 cultures; this had about 90 colonies compared to an average of less than one colony per streak. This culture gave an Hfr strain after sib selection. We also used sib selection on the three next most fertile cultures, each of which gave five colonies in the streak. All three showed an increased fertility in tube matings and we were able to isolate a second Hfr strain from one of the cultures.

Both Hfr strains were sensitive to ultraviolet light. They both transferred the chromosome efficiently in the order *metE, leu, trp. leu* was transferred at a similar efficiency to that in the *rec*$^+$ Hfr strains that we isolated. Most of the Leu$^+$ progeny were F$^-$. Thus the strains appear to be Hfr strains formed in a *recA* cell.

Reconstruction experiments give the mating efficiency in plate matings as about 0.7 progeny per Hfr cell; 8/449 streaks had more than three progeny colonies and if we assume that these cultures contained Hfr clones we obtained a value of 4.6×10^{-8}/cell/generation for the rate of Hfr formation. Thus, the rate of Hfr formation is about 100-fold less than that in the *rec*$^+$ strain, ED879.

Type II rec$^+$ *Strain*

We also performed fluctuation tests to verify the Type II nature of the *rec*$^+$ strain ED957. Table 3 shows that in the first experiment (test 3) the variation in fertility between samples in different tubes is only slightly larger than that of the control bulk culture (test 4), and could probably be accounted for by variation in the number of cells in each tube culture. However, in a further experiment (test 5) there was a large variance, due to two cultures with 288 and 82 progeny. Curtiss and Renshaw (1969a) had also obtained occasional fluctuations. We attempted to isolate Hfr clones from these cultures by sib selection. However, in such attempts we never obtained better than a twofold increase in the fertility of the cultures. This suggests that some high-fertility state may exist in this strain but that it is unstable or has an adverse effect on the growth rate of the host cell.

Type II recA Strain

In a fluctuation test, parallel cultures of ED969 did not show any significant variation in fertility. We therefore used streak matings to screen 900 cultures. None of these proved highly fertile in the streak matings. However, when we subcultured the 26 most fertile cultures and performed the more quantitative tube matings, these cultures gave an average of nearly 30 progeny colonies (minimum of 12) compared with about one progeny colony per tube from an ED969 bulk control culture. Thus, more fertile cultures do arise in the Type II *recA* strain. However, when we attempted sib selection we encountered similar problems to those with the Type II *rec*$^+$ strain. Thus, the Type II *recA* case is qualitatively similar to the Type II *rec*$^+$ case, but the fertility is much lower.

DISCUSSION

Table 2 shows that the efficiency of transfer of chromosomal genes from *recA* F$^+$ strains is about 100-fold lower than from *rec*$^+$ F$^+$ strains. This is comparable to the value of 3% reported by Clowes and Moody (1966). This reduction is not due to a general reduction in mating efficiency as transfer of F occurs at a similar frequency from the *rec*$^+$

and *recA* strains (Table 2). However, Table 2 shows that the apparent fertility for F transfer of the *recA* strains is greater than that of the *rec*$^+$ strains as a similar proportion of recipients become F$^+$ while the donor:recipient ratio is about half as large in the *recA* strains as the *rec*$^+$ strains. This may be due to nonviable cells of the *recA* strain that can transfer F but cannot form colonies.

We found little difference in the proportion of chromosomal recombinants that were F$^-$ between the *rec*$^+$ and *recA* strains, which suggested that the proportion of transfer due to Hfr cells, which give mainly F$^-$ progeny, was similar in the *rec*$^+$ and *recA* cases. This was confirmed by our finding that the rate of Hfr formation was also reduced about 100-fold in the *recA* strain compared to the *rec*$^+$ strain. The value of about 3×10^{-6}/cell/generation that we obtained for the *rec*$^+$ case was similar to that found by Curtiss and Stallions (1969). We were unable to use their method for the *recA* strain as this would have involved screening too many colonies (10^6–10^7).

Thus, our results show that Hfr formation and fertility in F$^+$ cultures depend to the same extent on the *recA* function. This rules out models that explain the depression of fertility in *recA* F$^+$ strains by the loss of only a non-Hfr component of fertility. It does not necessarily imply, however, that F interactions with the chromosome to form Hfr strains and to promote non-Hfr transfer have a common step. The chromosomal recombinants that are F$^+$ might arise from transfer of F occurring independently of chromosome transfer from the same donor cell, as suggested by Curtiss and Renshaw (1969b).

Like Curtiss and Renshaw (1969a), we were unable to isolate Hfr strains from a Type II *rec*$^+$ strain. However, occurrence of F$^-$ progeny at the same frequency as in matings with Type I donors (Curtiss and Renshaw, 1969b; this paper), and the existence of cultures with increased fertility in both the *rec*$^+$ and *recA* Type II strains suggested that some Hfr formation might occur in Type II strains. The failure of sib selection implied that this Hfr state was either unstable or disadvantageous. Thus, Type II strains did not seem to be a useful tool for investigating the contribution of Hfr formation to transfer by F$^+$ populations.

The question arises of whether the residual Hfr formation in *recA* strains is qualitatively different from the normal *recA*-dependent process. Excision in *recA* strains appears to involve nonhomologous recombination and gives rise to deletions and F′ formation (Deonier and Mirels, 1977). Broda and Meacock (1971) showed that it was possible for an F′*lac* to integrate within the *tsx* gene in a *recA* strain. Perhaps F can act as a transposon in *recA* strains; one possible model would be for the part of F between the two IS3 sequences (also known as $\alpha\beta$) to insert into the chromosome, giving a deletion in F between 0 and 15 kb (see map given by Deonier and Mirels, 1977). Such models could be tested using heteroduplex mapping.

ACKNOWLEDGMENTS

We thank the Medical Research Council for a Training Grant (to J.C.) and a Project Grant (to P.B.). We thank Helen Grozier for technical assistance.

REFERENCES

ALFARO, G., AND WILLETTS, N. (1972). The relationship between the transfer systems of some bacterial plasmids. *Genet. Res. Camb.* **20,** 279–289.

BACHMANN, B. J. (1972). Pedigrees of some mutant strains of *Escherichia coli* K12. *Bacteriol. Rev.* **36,** 525–557.

BRODA, P. (1967). The formation of Hfr strains in *Escherichia coli* K12. *Genet. Res. Camb.* **9,** 35–47.

BRODA, P., AND MEACOCK, P. (1971). Isolation and characterisation of Hfr strains from a recombination-deficient strain of *Escherichia coli*. *Mol. Gen. Genet.* **113,** 166–173.

CAMPBELL, A. (1962). Episomes. *Adv. Gen.* **11,** 101–146.

CLOWES, R. C., AND MOODY, E. E. M. (1966). Chromosomal transfer from "recombination-deficient" strains of *Escherichia coli* K12. *Genetics* **53,** 717–726.

CULLUM, J., COLLINS, J. F., AND BRODA, P. (1978) Factors affecting the kinetics of progeny formation with F′*lac* in *Escherichia coli* K12. *Plasmid* **1,** 536–544.

CURTISS, R. III, AND RENSHAW, J. (1969a). F$^+$ strains

of *Escherichia coli* K12 defective in Hfr formation. *Genetics* **63**, 7–26.

CURTISS, R. III, AND RENSHAW, J. (1969b). Kinetics of F transfer and recombinant production in $F^+ \times F^-$ matings in *Escherichia coli* K12. *Genetics* **63**, 39–52.

CURTISS, R. III, AND STALLIONS, D. R. (1969). Probability of F integration and frequency of stable Hfr donors in F^+ populations of *Escherichia coli* K12. *Genetics* **63**, 27–38.

DEONIER, R. C., AND DAVIDSON, N. (1976). The sequence organization of the integrated F plasmid in two Hfr strains of *Escherichia coli*. *J. Mol. Biol.* **107**, 207–222.

DEONIER, R. C., AND MIRELS, L. (1977). Excision of F plasmid sequences by recombination at directly repeated insertion sequence 2 elements: Involvement of *recA*. *Proc. Natl. Acad. Sci. USA* **74**, 3965–3969.

DE VRIES, J. K., AND MAAS, W. K. (1971). Chromosomal integration of F′ factors in recombination-deficient Hfr strains of *Escherichia coli*. *J. Bacteriol.* **106**, 150–156.

HU, S., OHTSUBO, E., AND DAVIDSON, N. (1975). Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*: Structure of F13 and related F-primes. *J. Bacteriol.* **122**, 749–763.

JACOB, F., AND WOLLMAN, E. L. (1956). Recombinaison génétique et mutants de fertilité chez *Escherichia coli*. *C.R. Hebd. Seanc. Acad. Sci., Paris* **242**, 303–307.

LURIA, S. E., AND DELBRÜCK, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**, 491–511.

OHTSUBO, E., DEONIER, R. C., LEE, H. J., AND DAVIDSON, N. (1974). Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. IV. The F sequences in F14. *J. Mol. Biol.* **89**, 565–584.

REEVES, P. (1960). Role of Hfr mutants in $F^+ \times F^-$ crosses in *Escherichia coli* K12. *Nature (London)* **185**, 265–266.

STARLINGER, P., AND SAEDLER, H. (1976). IS-elements in microorganisms. *Current Topics Microbiol. Immunol.* **75**, 111–152.

(Reprinted from Nature New Biology, Vol. 232, No. 31, pp. 137–140, August 4, 1971)

Evidence for the Bidirectional Replication of the *Escherichia coli* Chromosome

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Experiments with transducing phage indicate that the circular chromosome of *Escherichia coli* is replicated in both directions from a fixed origin.

E chromosome of *Escherichia coli* is a single, circular DNA olecule which replicates sequentially[1–3] from a fixed ori-[4–14] which has been located within a region representing out 20% of the length of the chromosome between 50 and minutes on the genetic map[15] (Fig. 1). Although it has been ought that replication proceeds in a unique direction, we w describe experiments which suggest that replication takes ce not in one but in both directions from an origin at about minutes on the Taylor map to a terminus separated from it approximately 30% of the length of the chromosome.

easuring Gene Frequency

n *E. coli*, the generalized transducing phage P1 can be d as a vector of genetic material. P1 transduces each cterial gene with a characteristic frequency, presumably ecting both the relative frequency of the gene and its lity to be integrated. Independently, one of us[8] and Caro l Berg[4] have devised a method for using P1 to measure e frequency in which variability in expression is compen-ed for by comparing the transducing activities only of pairs ysates, each consisting of one lysate from a rapidly growing ture and one from a slowly growing culture. The chromo-nes of rapidly growing bacteria replicate dichotomously l, because they have three replication forks, they have four gin markers for each terminus marker. Because chromo-nes of slowly growing bacteria have only one replication k and two origin markers per terminus marker, the ratios transducing activities for a number of markers in the two ates should decrease from a value of 2 for a marker at the gin of replication to 1 for a marker at the terminus (see . 17 and 18).

Fig. 2 shows the marker frequency ratios determined for in B/r and for strain K12 by this method, plotted against genetic map positions of the markers. The points fall on urve with a gradient decreasing in both directions from a ximum at about 60 minutes. The simplest interpretation these data is that replication proceeds bidirectionally from xed origin at this maximum to a terminus elsewhere on chromosome.

The absolute numbers of transductants obtained for different rkers in single P1 lysates also fall on a bidirectional gradient ted to gene position. This is what one would expect if the frequencies of transductants depended primarily on the position of the markers on the chromosome, rather than on integration and expression parameters characteristic of individual markers. We therefore analysed the transducing activities of individual lysates more precisely to see if this simpler procedure would lead to the same conclusions about chromosome replication as did the ratios of marker frequencies in pairs of lysates.

Gene Frequencies in F⁻ Strains

Three F⁻ strains of *E. coli*, the K12 strains W1485 and W3110, and strain B/r, were used as donors of genetic material. Lysates were prepared from cultures growing in broth and

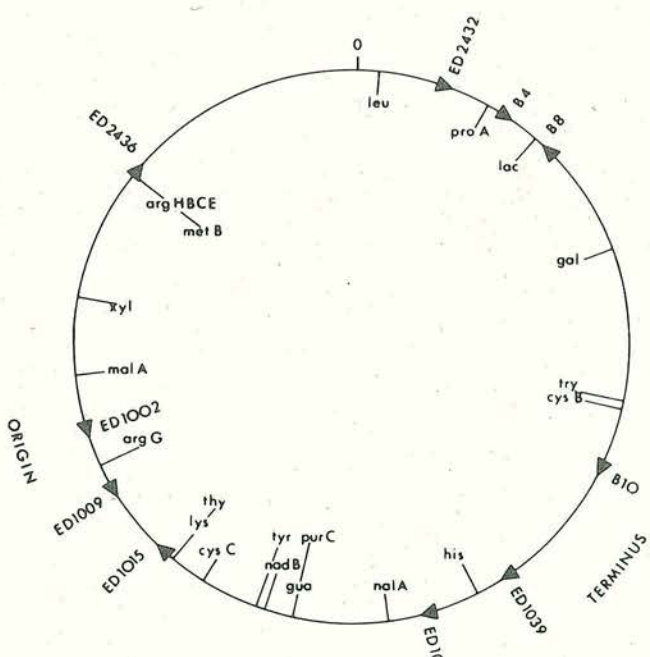


Fig. 1 Genetic map of *E. coli* after Taylor[15], showing the location of markers used in transduction experiments, the origin and direction of transfer of the Hfr strains that were used, and the location of the origin and terminus of vegetative replication. The nomenclature used is that of Taylor[15]. The characterization of the B series of Hfr strains has been described previously[16]. The origins and directions of transfer of the ED series of Hfr strains were determined by crosses against appropriate multiply marked recipient strains. They were then confirmed in interrupted mating experiments.

dividing every 20 min (W1485 and B/r) or every 25 min (W3110). These lysates were used to transduce a series of B/r (ref. 8) and K12 recipient strains (Fig. 2). Among the markers of K12 recipient strains is one, either an arginine marker of the group *argHBCE*, or *metB*, located at 77 minutes on the genetic map (Fig. 1). Frequencies of transduction for different markers in different experiments and using different recipients were normalized against the number of *arg*$^+$ or *met*$^+$ transductants. In B/r transductions, frequencies were normalized by setting the number of *thy*$^+$ transductants equal to 1.5. The markers which were used in these transduction experiments are shown in Fig. 1.

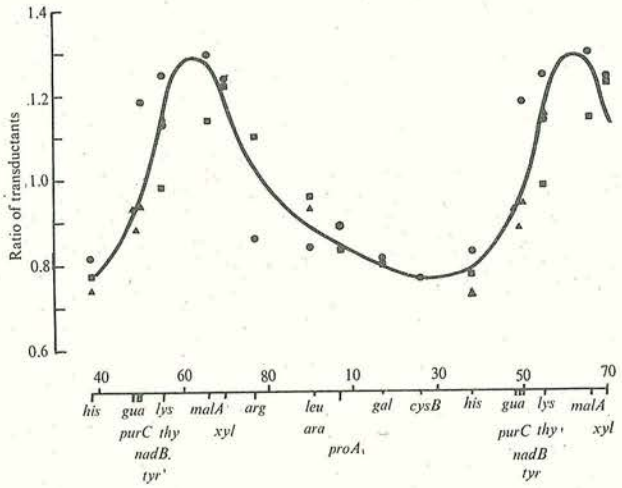


Fig. 2 The ratios of the relative numbers of transductants in broth and minimal lysates for a series of markers in three F$^-$ strains of *E. coli*, ▲ B/r, ■ W1485, ● W3110. Lysates were prepared and transductions carried out as previously described[8]. Frequencies were normalized as described in the text. The curves obtained for each of the three strains were combined to obtain the composite curve of best fit. The recipient strains used in the transductions were AB2147 (*lacY gal his malA xyl ilv argH metB thi* (P1)$^+$), JC5422 (*ara thr leu proA lac gal his thy str xyl mtl argE thi*), AT2273 (*lac gal his tyr malA xyl arg* thi*), X36 (*lac gal trp pyrF his purC str malA xyl arg* thi*), JC411 (*leu lac gal his argG str malA xyl mtl metB*) and derivatives of these. The asterisk indicates one of the group located at 77 minutes.

Fig. 3 shows the frequencies of transductants obtained with these lysates; the frequencies depend on gene position. Thus the frequency of transductants observed for a particular marker seems to be a function of the position of the marker on the bacterial chromosome and is not greatly influenced by marker-dependent variation in the frequency of integration and/or expression. The highest frequency obtained was that for *malA*, which is located at 66 minutes on the genetic map. The other markers fall on a bidirectional gradient, reaching a minimum with *his*. Because the maximum and minimum are similar to those of the gene frequency ratio curve, we conclude that they too represent the origin and terminus of chromosome replication.

Gene Frequencies in Isogenic Male Strains

There is disagreement about whether vegetative replication in Hfr strains of *E. coli* originates from the site of integration of the sex factor F[4,5,13,19–21]. We have examined three Hfr strains (B4, B8 and B10) and their F$^+$ parent W1655 to compare the transduction frequency distributions with those found for F$^-$ strains. The origins and directions of transfer of these Hfr strains are given in Fig. 1. One of these strains (Hfr B4) has previously been reported[13] to have an origin of replication near to the site of the integrated sex factor. In contrast and in confirmation of the observations of Berg and Caro[5] on another series of isogenic Hfr strains, we found that the frequency distribution of transductants in all four strains was the same as that observed in the F$^-$ strains (Fig. 3). The integration of the sex factor in Hfr strains therefore does not seem to influence transduction frequencies or the origin of replication.

Gene Frequencies in Hfr *lac*$^+$ Transposition Strains

Our experiments demonstrate that the position of a marker on the chromosome determines the frequency with which it is transduced. We have isolated Hfr strains by integration of a temperature-sensitive F-prime factor (F$_{ts114}$*lac*$^+$) at different locations in the chromosome of a strain carrying a *lac-pro* deletion (ED903: ▽*lac-pro*$_{x111}$). These strains have a single *lac*$^+$ region which, in each Hfr strain, is at the site at which the sex factor is integrated. Transducing lysates were prepared from these strains and the frequencies with which *lac*$^+$ was transduced by the different lysates were compared with each other and with the transduction frequencies of other markers. The results are shown in Fig. 4. We found, first, that the relative frequencies of transduction for markers other than *lac* were similar for all seven classes of Hfr studied. Thus, once again, the site at which the sex factor is integrated has no effect on the frequency of transduction. Second, the frequency of *lac*$^+$ transductants from each Hfr strain is a function of the chromosomal position of the *lac*$^+$ genes. The fact that a single gene, *lac*$^+$, is transduced with different frequencies depending on its position on the chromosome confirms our conclusion that the relative frequency of transduction observed for a particular marker is a function of its position on the chromosome.

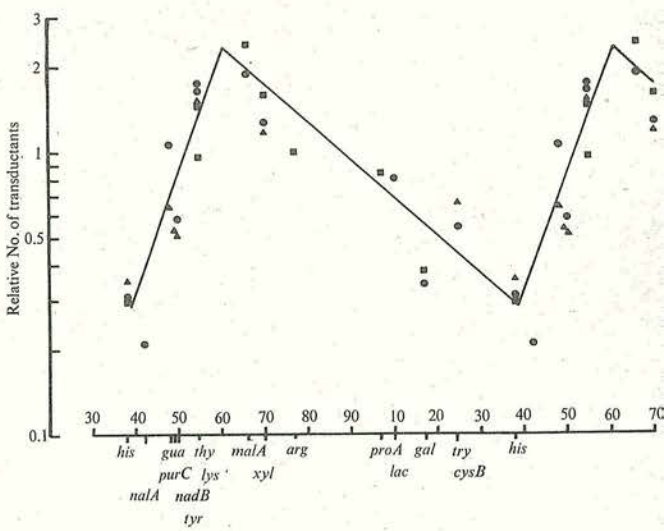
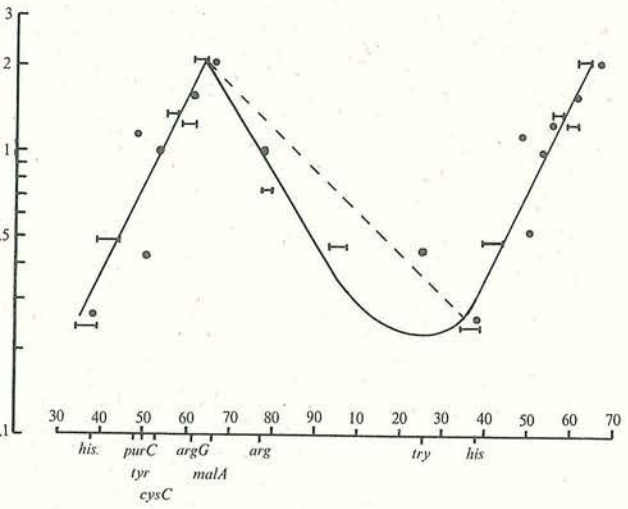


Fig. 3 Transductant frequency distribution obtained from broth lysates of three F$^-$ strains of *E. coli*, ▲ B/r, ■ W1485, ● W3110. Frequencies were normalized as described in the text.

The solid curve in Fig. 4 is drawn to fit the frequencies with which *lac*$^+$ is transduced. The portion between 35 and 65 minutes is identical to the corresponding portion of the curves in Fig. 3. But the *lac* transpositions between 65 and 35 minutes define a more steeply descending curve than do the other markers used (dashed line). If the curve defined by the *lac* transpositions is a better measure of gene frequency than the curve in Fig. 3, the minimum of the frequency distribution would be further to the left, between 20 and 25 minutes. This is

e same minimum as is found in the curve of frequency ratios
g. 2).

In most strains examined, the highest frequency of transction was obtained for the marker *malA*. The sole exception s one of the transposition strains, strain ED1002, in which e F′ factor is integrated between *malA* and *argG*. In this ain the frequency of *lac*$^+$ transductants was higher than it for *malA*$^+$. We conclude that the peak in transduction quency and the origin of replication of the chromosome ls between *malA* and *argG*.



ig. 4 Tranductant frequency distribution in broth lysates f several *lac*-transposition Hfr strains. ⊢, *lac*$^+$ transductants, lotted as a function of the position of the *lac*$^+$ region in the onor chromosome; the horizontal line indicates that *lac*$^+$ lls within the region delineated. ●, Transductants for other arkers; each point represents the averaged value from the seven donor strains.

irectional Replication

oth marker frequency ratios (Fig. 2) and relative transing frequencies in individual lysates (Figs. 3, 4) decrease oth directions from a maximum between 60 and 65 minutes a minimum between 25 and 40 minutes. The simplest rpretation of our observations is that these points are the in and terminus of replication, and that because they are eparate locations on the chromosome, replication proceeds rectionally from the origin to the terminus. DNA replica- is bidirectional in phage λ[22] and in eukaryotic cells[23]. hioka and Eisenstark[24] have found that markers from osite sides of the chromosome were synthesized apparently ng the middle of the chromosome replication cycle of *honella typhimurium* and therefore concluded that the omosome of this close relative of *E. coli* replicates bidirecally. Much of the published evidence concerning DNA ication in *E. coli* is consistent with bidirectional replication n a fixed origin[1,4,6–8,11,13,14], although it has not presly been interpreted in this way. The best known of this k is that of Cairns[1], who concluded from his autoradiohs of replicating DNA molecules that replication proceeds irectionally. He reasoned that one of the two forks erved in such molecules was the point of replication and other the origin of replication. If replication is bidirecal each of these forks would represent a replication site. interpretation would be consistent with the labelling ern observed at the forks by Cairns only if bidirectional cation is not entirely symmetrical, either on the level of vidual molecules only or for the population as a whole. n asymmetric replication has been observed in individual replicating molecules of phage λ[22] and is suggested by our data for the whole cell population of *E. coli* (see discussion below).

In contrast, in *Bacillus subtilis*, the one other bacterial species which has been intensively studied[17,18,25–27], there is no evidence to suggest that replication is not unidirectional.

Is Bidirectional Replication Symmetrical?

Our experiments suggest that the best estimate for the position of the origin of replication is between 60 and 65 minutes. The terminus is less well defined and seems to be between 25 and 40 minutes. If the current genetic map accurately represents physical distance on the chromosome, then the distance from the origin to the terminus is greater in the clockwise direction than in the counterclockwise one and therefore replication is not symmetrical. Although gene frequencies in individual lysates indicate that the terminus is near to 40 minutes (Fig. 3), both the results obtained with the *lac* transposition strains (Fig. 4) and the frequency ratio experiments (Fig. 2) favour a terminus of replication near to 25 minutes. In this case replication would be approximately symmetrical. Examination of further *lac* transposition strains, with the *lac*$^+$ gene inserted between 5 and 25 minutes, is perhaps the most promising method for more exact location of the terminus.

If replication is asymmetric, this could be the result either of a difference in the rate at which it proceeds in the two directions, or of a difference in the time that it proceeds (as would be the case if the terminus were in some way fixed). In the latter case, the rate of DNA synthesis in synchronous cultures would halve at some time during the cell cycle. As this has not been observed[28] this alternative can probably be excluded.

Frequency Distribution of Transduced Markers

We have shown that the frequencies of transduction of genes on the *E. coli* chromosome are a function of the relative frequencies of those genes in the donor gene population. But if transducing particles incorporate bacterial DNA at random, so that the frequency gradient actually represents the relative frequencies of genes in the donor gene population, the total range of the gradient, in a culture where DNA is being replicated dichotomously, should be no greater than 4 : 1. As can be seen from Figs. 3 and 4, the actual range in frequencies is about eight-fold; this would not be expected in cells with fewer than an average of seven replication forks per chromosome. Although such chromosomes occur when, or after, the rate of DNA synthesis at individual replication forks is restricted[29,30] this was not the case in these experiments.

An alternative hypothesis is that phage P1 does not incorporate bacterial DNA randomly but has a gradient of preference in incorporation. Some preferential incorporation of origin markers, rather than terminus markers, into transducing particles could account for the 8 : 1 gradient observed in transduction frequency.

While this article was being written we learned that Bird, Louarn and Caro (personal communication) have come to similar conclusions using an entirely different technique. They suggest that the origin lies at 74 minutes and the terminus at 25 minutes.

We thank Derek Harland and Peter Meacock for technical assistance, and W. D. Donachie for encouragement.

Received May 10; revised July 2, 1971.

[1] Cairns, J., *J. Mol. Biol.*, 6, 208 (1963).
[2] Meselson, M., and Stahl, F. W., *Proc. US Nat. Acad. Sci.*, 44, 671 (1958).
[3] Lark, K. G., Repko, T., and Hoffman, E. J., *Biochim. Biophys. Acta*, 76, 9 (1963).

[4] Caro, L. C., and Berg, C. M., *J. Mol. Biol.*, **45**, 325 (1969).
[5] Berg, C. M., and Caro, L. C., *J. Mol. Biol.*, **29**, 419 (1967).
[6] Donachie, W. D., and Masters, M., *Genet. Res. Camb.*, **8**, 119 (1966).
[7] Donachie, W. D., and Masters, M., in *The Cell Cycle: Gene-Enzyme Interactions* (edit. by Padilla, Whitson and Cameron), 37 (Academic Press, New York and London, 1969).
[8] Masters, M., *Proc. US Nat. Acad. Sci.*, **65**, 601 (1970).
[9] Helmstetter, C., *J. Bact.*, **95**, 1634 (1968).
[10] Pato, M. L., and Glaser, D., *Proc. US Nat. Acad. Sci.*, **60**, 1268 (1968).
[11] Abe, M., and Tomizawa, J., *Proc. US Nat. Acad. Sci.*, **58**, 1911 (1967).
[12] Cerdá-Olmedo, E., Hanawalt, P. C., and Guerola, N., *J. Mol. Biol.*, **33**, 705 (1968).
[13] Wolf, B., Newman, A., and Glaser, D., *J. Mol. Biol.*, **32**, 611 (1968).
[14] Ward, C. B., and Glaser, D. A., *Proc. US Nat. Acad. Sci.*, **62**, 881 (1969).
[15] Taylor, A. L., *Bact. Rev.*, **34**, 155 (1970).
[16] Broda, P., *Genet. Res. Camb.*, **9**, 35 (1967).
[17] Yoshikawa, H., O'Sullivan, A., and Sueoka, N., *Proc. US Nat. Acad. Sci.*, **52**, 973 (1964).
[18] Sueoka, N., and Yoshikawa, H., *Genetics*, **52**, 748 (1965).
[19] Nagata, T., *Proc. US Nat. Acad. Sci.*, **49**, 551 (1963).
[20] Nishi, A., and Horiuchi, T., *J. Biochem.*, **60**, 338 (1966).
[21] Vielmetter, W., Messer, W., and Schütte, A., *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 585 (1968).
[22] Schnös, M., and Inman, R. B., *J. Mol. Biol.*, **51**, 61 (1970).
[23] Huberman, J. A., and Riggs, A. D., *J. Mol. Biol.*, **32**, 327 (1968).
[24] Nishioka, Y., and Eisenstark, A., *J. Bact.*, **102**, 320 (1970).
[25] Yoshikawa, H., and Sueoka, N., *Proc. US Nat. Acad. Sci.*, **49**, 559 (1963).
[26] Yoshikawa, H., and Sueoka, N., *Proc. US Nat. Acad. Sci.*, **49**, 806 (1963).
[27] Dubnau, D., Goldthwaite, C., Smith, I., and Marmur, J., *J. Mol. Biol.*, **27**, 163 (1967).
[28] Helmstetter, C. E., and Cooper, S., *J. Mol. Biol.*, **31**, 507 (1968).
[29] Donachie, W. D., *J. Bact.*, **100**, 260 (1969).
[30] Pritchard, R. H., and Zaritsky, A., *Nature*, **226**, 126 (1970)

Printed in Great Britain by Flarepath Printers Limited, Watling Street, Colney Street, St. Albans, Herts.

Molec. gen. Genet. 127, 255—258 (1973)

DNA Polymerase III and the Replication of F and ColVB*trp* in *Escherichia coli* K-12

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Received October 5, 1973

Summary. In an *E. coli* K-12 strain producing a thermolabile, mutagenic DNA polymerase III, enhanced reversion rates were observed for a *trp* missense mutation on F'*trp* and ColVB*trp*. In addition, replication of these plasmid DNA molecules was greatly reduced at the non-permissive temperature. Both observations indicate that DNA polymerase III is involved in the replication of these plasmids.

Introduction

The product of the *dnaE* gene of *Escherichia coli* K-12, DNA polymerase III (Gefter *et al.*, 1971), is essential for chromosome replication *in vivo* (Wechsler and Gross, 1971) and *in vitro* (Nüsslein *et al.*, 1971). Goebel (1972) has shown that the transmissible plasmids ColV(K30), ColIb(P9) and Hly are not replicated in a temperature-sensitive (*ts*) *dnaE* mutant at the non-permissive temperature, whereas the replication of the small non-transmissible plasmid $ColE_1$ continues.

We have examined the role of *dnaE* in plasmid replication under conditions in which chromosome replication and cell growth are normal. Some *ts* alleles of *dnaE* are mutagenic at permissive temperatures (Hall and Brammar, 1973). We have studied the effect of one such allele on the reversion rate of a *trpA* point mutation carried by two plasmids, F'*trp* and ColVB*trp*. In addition, we have examined plasmid DNA synthesis at the non-permissive temperature. The results of both types of experiment indicate that DNA polymerase III is involved in the replication of these two transmissible plasmids.

Materials and Methods

Construction of Strains. The plasmids KLF23 (Low, 1972) and ColVB*trp* (Frédéricq, 1969), both of which carry the chromosomal genes $cysB^+$, $tonB^+$ and trp^+, were transferred to strain WB51 (W3110 *cysB tonB-trpAE*$_{del}$) (Hall and Brammar, 1973). Spontaneously homogenotised Cys^- derivatives were selected after penicillin enrichment. The *trpA58* allele (Hall and Brammar, 1973) was introduced from a $cysB^+$ *trpA58* donor by transduction with phage P1kc, making use of the linkage between $cysB^+$ and *trp* (Yanofsky and Lennox, 1959). Cys^+ transductants were screened for Trp^- clones. Since chromosomal Cys^+ transductants remain Trp^+ due to the presence of the trp^+ plasmid, Trp^- transductants are recombinant in the plasmid. The presence of $cysB^+$ on the plasmids was confirmed by their ability to transfer $cysB^+$ to strain W3110 *cysB tonB-trpAE*$_{del}$ *str*. Since such $CysB^+$ progeny were able to grow on minimal plates supplemented with indole (10 μg/ml), and accumulate indole-glycerol when grown in limiting tryptophan (Allen and Yanofsky, 1963) these plasmids were also *trpA*. The genetically marked plasmids were stored in a $recA^-$ host ,and subsequently transferred to strain W3110 *cysB tonB-trpA*$_{del}$ *tonA dnaE486* (Hall and Brammar, 1973) and the isogenic $dnaE^+$ strain to give the strains listed in Table 1.

Table 1. Reversion of extrachromosomal *trpA58* allele in *dnaE ts* and wild type backgrounds

Strain	*dnaE* allele	Plasmid carried	Phenotypic reversion frequency	Plasmid reversion frequency
ED3551	486	ColVB*trpA58*	445	351
ED3552	+	ColVB*trpA58*	3.0	2.8
ED3558	486	KLF23*trpA58*	439	323
ED3559	+	KLF23*trpA58*	2.9	2.6

Frequencies are expressed as revertants/10^9 cells. They are the means of 3–5 experiments.

Reversion to Trp$^+$. Cultures from single colonies were grown overnight at 30° C in Oxoid broth supplemented with tryptophan (4 μg/ml). Cells were harvested by centrifugation, washed in M9 buffer, concentrated tenfold and 0.1 ml samples were spread on plates selective for Trp$^+$ revertants. Viable counts were performed on similar plates supplemented with tryptophan (800 μg/plate). Incubation was for 4–5 days at 30° C. Phenotypically revertant colonies were patched and plate mated with a *recA*$^-$ strain to demonstrate that the plasmid markers were independent of the chromosome, and with a Sm^R Trp_{del} strain to establish whether the reversion event had occurred on the chromosome or on the plasmid. Revertants able to give Trp$^+$ progeny with the Trp_{del} recipient strain were scored as carrying revertant plasmids.

Isolation of Plasmid DNA. Cells were grown at 30° C in M9-glucose minimal medium supplemented with 0.75% (w/v) casamino acids and 20 μg/ml tryptophan. Labelling was for 90′ at the indicated temperature with 10 μCi/ml ^{3}H-thymidine (21.5 Ci/mmol, Amersham, England) in the presence of deoxyadenosine (300 μg/ml, Sigma). Cleared lysates were prepared by the procedure of Clewell and Helinski (1971) with the modifications given below. The washed cell pellet was resuspended in 25% sucrose, 0.05 *M* Tris (pH 8.0) solution, and spheroplasts were prepared by the addition of lysozyme and EDTA. Lysis was accomplished by the addition of a solution containing 2% Triton X-100 (Sigma), 0.05 *M* Tris (pH 8.0) and 0.0625 *M* EDTA to an equal volume of spheroplasts. The resulting crude lysate was centrifuged at 27000 g for 15′; the pellet, containing the bulk of the chromosomal DNA, was discarded and 2 ml of the supernatant (the cleared lysate) was mixed with 0.5 ml ethidium bromide solution (2 mg/ml, Calbiochem) and 1.5 ml TES (0.05 *M* Tris, 0.05 *M* NaCl, 0.005 *M* EDTA, pH 8.0). The density was adjusted to 1.55 gm/cc with CsCl and the mixture was centrifuged for 40 hours at 40000 rpm and 20° C in an SW50.1 rotor of a Spinco model L2-50 centrifuge. Fractions were collected directly onto Whatman 3MM paper discs or into small tubes (for further analysis) from which samples were spotted onto discs. Dried discs were immersed in 5% (w/v) TCA, washed in 80% (v/v) ethanol, dried and counted for radioactivity.

Results and Discussion

Derivatives of KLF23 and ColVB*trp* carrying the *trpA58* mutation were constructed and introduced into *dna*$^+$ and *dnaE486* strains deleted for the *trp* operon; the Trp$^+$ reversion frequencies of these strains were then determined (see Materials and Methods). Patch matings with a *recA* recipient demonstrated that in both the initial cultures and the revertant clones, the plasmid markers were independent of the chromosome. The proportion of the Trp$^+$ revertants due to chromosomal suppressor mutations (Yanofsky *et al.*, 1966) was determined so that the frequency of reversion events on the plasmid DNA could be calculated. The reversion frequency in the presence of the *dnaE486* allele is greatly increased (Table 1). We conclude that the mutator activity of *dnaE486* acts on both KLF23 and ColVB*trp* DNA, and therefore that DNA polymerase III is involved in base selection during

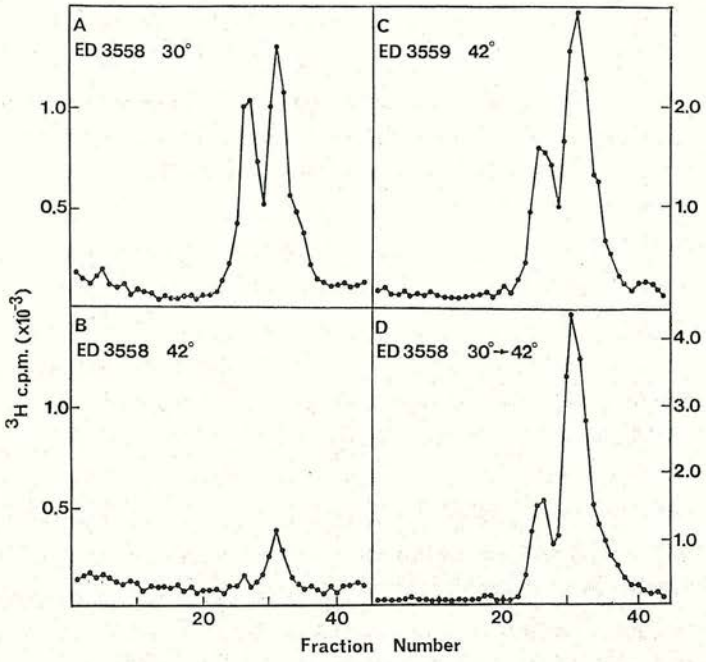

Fig. 1 A—D. CsCl-ethidium bromide density gradient centrifugation of cleared lysates of strains carrying KLF23*trpA58*. Strain ED3558 was labelled at 30° C (A) and 42° C (B); DNA prelabelled at 30° C was examined after 90′ incubation in unlabelled medium at 42° C (D). Strain ED3559 labelled at 42° C is shown in C. Density increases from right to left

plasmid DNA replication as well as during chromosome replication (Hall and Brammar, 1973).

In addition, we have examined the DNA synthesised at 30 and 42° C in the plasmid-carrying *dnaE486* strains. Cleared lysates of strain ED3558 grown and labelled at 30° C show two peaks on dye-buoyant density centrifugation (Radloff *et al.*, 1967) (Fig. 1, panel A). The denser peak, comprising supercoiled KLF23-*trpA58* DNA, gave a rapidly sedimenting (150S) peak on neutral sucrose gradients. The less dense peak was composed of chromosomal fragments. Synthesis of supercoiled KLF23*trpA58* molecules is greatly reduced at 42° C (Fig. 1, panel B) and no DNA characteristic of open circular molecules was detected when the light peak material was examined on neutral sucrose gradients. In contrast, the *dnaE*$^+$ strain can synthesise plasmid DNA efficiently at 42° C (Fig. 1, panel C). The possibility of extensive loss of supercoiled molecules during incubation at 42° C was excluded by the demonstration that plasmid DNA labelled at 30° C could be recovered in high yield after the cells were held at 42° C for 90′ (Fig. 1, panel D). Moreover, degradation of DNA from the replication fork does not occur in *dnaE486* cells at 42° C (Schuster *et al.*, 1973).

Both the reversion frequency experiments at the permissive temperature and the labelling experiments at the non-permissive temperature suggest that replication of KLF23*trpA58* and ColVB*trpA58* depends upon DNA polymerase III.

Whether this enzyme has a role in transfer is unclear. We have found no difference between *dna*$^+$ and *dnaE486* donors in their ability to transfer these plasmids at 42° C. Preincubation at 42 or 45° C for up to 120′ before mating also revealed no differences. However, in view of the leakiness of the *dnaE486* mutation that we observe and the low transfer frequencies found under these conditions (less than 20 progeny per 100 donor cells) it is not possible to exclude a role for DNA polymerase III in transfer.

We are grateful to W. J. Brammar and M. A. Rabstein for strains and advice.

References

Allen, M. K., Yanofsky, C.: A biochemical and genetic study of reversion with the A gene A protein system of *E. coli* tryptophan synthetase. Genetics **48**, 1056–1083 (1963)

Clewell, D. B., Helinski, D. R.: Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**, 4428–4440 (1971)

Frédéricq, P.: The recombination of colicinogenic factors with other episomes and plasmids. In: Bacterial episomes and plasmids (G. E. W. Wolstenholme and M. O'Connor, eds.), p. 163–174. London: J. & A. Churchill 1969

Gefter, M. L., Hirota, Y., Kornberg, T., Wechsler, J. A., Bernoux, C.: Analysis of DNA polymerases II and III in mutants of *Escherichia coli* thermosensitive for DNA synthesis. Proc. nat. Acad. Sci. (Wash.) **40**, 3150–3153 (1971)

Goebel, W.: Replication of the DNA of the colinicogenic factor E_1 (Col E_1) at the restrictive temperature in a DNA replication mutant thermosensitive for DNA polymerase III. Nature (Lond.) New Biol. **237**, 67–70 (1972)

Hall, R. M., Brammar, W. J.: Increased spontaneous mutation rates in mutants of *E. coli* with altered DNA polymerase III. Molec. gen. Genet. **121**, 271–276 (1973)

Low, K. B.: *Escherichia coli* K-12 F-prime factors, old and new. Bact. Revs. **36**, 587–607 (1972)

Nüsslein, V., Otto, B., Bonhoeffer, F., Schaller, H.: Function of DNA polymerase III in DNA replication. Nature (Lond.) New Biol. **234**, 285–286 (1971)

Radloff, R., Bauer, W., Vinograd, J.: A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in Hela cells. Proc. nat. Acad. Sci. (Wash.) **57**, 1514–1521 (1967)

Schuster, H., Beyersmann, D., Mikolajczyk, M., Schlicht, M.: Prophage induction by high temperature in thermosensitive *dna* mutants lysogenic for bacteriophage lambda. J. Virol. **11**, 879–885 (1973)

Wechsler, J. A., Gross, J. D.: *Escherichia coli* mutants temperature-sensitive for DNA synthesis. Molec. gen. Genet. **113**, 273–284 (1971)

Yanofsky, C., Ito, J., Horn, V.: Amino acid replacements and the genetic code. Cold Spr. Harb. Symp. quant. Biol. **31**, 151–162 (1966)

Yanofsky, C., Lennox, E. S.: Transduction and recombination study of linkage relationships among the genes controlling tryptophan synthesis in *Escherichia coli*. Virology 8, 425–447 (1959)

Communicated by Ch. Auerbach

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Reprinted from *J. Mol. Biol.* (1974) **89**, 647–650

Electron Microscope Heteroduplex Studies of Sequence Relations Among Plasmids of *Escherichia coli*

IX. Note on the Deletion Mutant of F, FΔ(33-43)

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J. Mol. Biol. (1974) 89, 647–650

Electron Microscope Heteroduplex Studies of Sequence Relations Among Plasmids of *Escherichia coli*

IX.† Note on the Deletion Mutant of F, FΔ(33-43)

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Heteroduplex analysis shows that the plasmid extracted from cells of the F$^+$ strain, W1655, is a deletion mutant of F, and lacks segments with F co-ordinates 32·6 to 42·9 kilobases. The genes on F for resistance to the female-specific phages T3, ϕII, and τ probably lie within this region. Consideration of the sequences deleted in several F-primes shows that the sequences from 0 to 42·9 kilobases on F are not necessary for autonomous replication nor for fertility.

1. Introduction

We wish to report that the F plasmid present in cells of an F$^+$ strain, W1655, used in some previous studies (Broda, 1967) is a deletion mutant of F. It is missing the sequences of F from 32·6 to 42·9 kb‖ in the co-ordinate system that we have introduced for the F factor (Sharp *et al.*, 1972), and is therefore designated FΔ(33-43). It is useful as a reference molecule in heteroduplex studies of other F-primes.

2. Materials and Methods

Strain W1655, which is *metB*$^-$ λ^- λ^r, was derived from *Escherichia coli* K12 in a series of steps, some of which were mutagenic (see chart 5 of Bachmann, 1972). It carries a transmissible F plasmid that is mutant, in that it does not confer upon its host resistance to

† This is one of a series of accompanying papers. Paper VIII is Ohtsubo *et al.* (1974*b*).

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‖ Abbreviation used: kb, kilobases, thousands of bases or base-pairs for single strand and duplex DNA, respectively.

phage T3, which plates with low efficiency on most strains carrying F (Schell *et al.*, 1963)† phage ϕII and phage τ (see Results). JE3100, bearing the plasmid F8-2, has been described elsewhere. JE2571 is the isogenic F^- progenitor of JE3100 (Ohtsubo *et al.*, 1970; Sharp *et al.*, 1972; Ohtsubo & Hsu, 1974). JE102 F^- is a lysogen of phage τ (Hakura *et al.*, 1964).

The methods of plasmid extraction and electron microscopy that we used have been described by Sharp *et al.* (1972) and Ohtsubo *et al.* (1974).

Sensitivity to phage τ was tested as follows. Several 5-ml samples of a T-broth culture of JE102 at a cell density of $1{\cdot}5 \times 10^7$ cells/ml were induced by exposure to u.v. light and incubated at 37°C until the onset of lysis. The remaining bacteria were lysed by adding chloroform. Dilutions (1:10, 1:50, and 1:100) of the lysates were streaked on T-broth agar plates. The bacteria to be tested were then cross-streaked on the plates.

3. Results

In anticipation of the results reported below, we designate the plasmid extracted from W1655 as FΔ(33-43). Its length, as measured by spreading the open circular DNA by the aqueous technique, was observed to be shorter than that of our standard F from W1485 by $10 \pm 2\%$. A careful, direct comparison with F gives a value of $0{\cdot}887 \pm 0{\cdot}016$ for the length ratio, suggesting that the molecular weight of FΔ(33-43) is $83{\cdot}8 \pm 1{\cdot}6$ kb. (F is taken as 94·5 kb (Sharp *et al.*, 1972).)

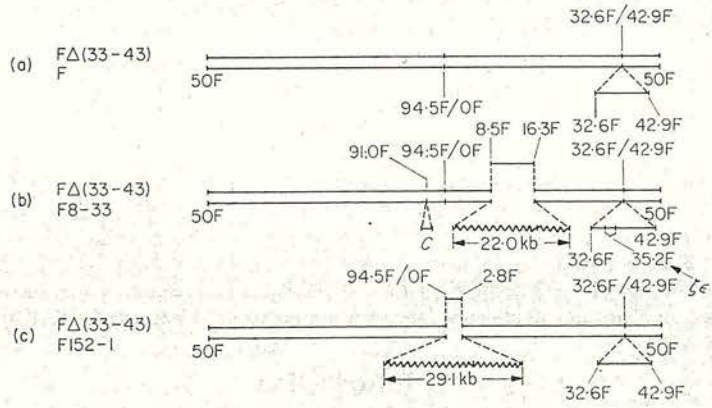

FIG. 1. Heteroduplexes used for the structure determination of FΔ(33-43) as discussed in the text. Note that circular molecules are represented in a linear form by cutting the duplex at the point 50F, which lacks interesting features.

The structure of the plasmid was deduced from a study of its heteroduplexes with F and with the reference F-primes, F8-33 and F152-1. The structures of these latter two reference F-primes are described elsewhere (Ohtsubo & Hsu, personal communication; Sharp *et al.*, 1972).

The observed structures of the heteroduplexes of FΔ(33-43) are depicted in Figure 1. Electron micrographs of heteroduplexes with F and with F152-1 are shown in Plate I.

The structure observed for the F/FΔ(33-43) heteroduplex shows that the latter molecule is a simple deletion mutant of F, but it does not map the position of the

† Strain W1655 was referred to mistakenly by Schell *et al.* (1963: Table 1, cross number 10) as strain W1485. The F factor from the true strain W1485 is not deleted in the 33-43 kb region (Sharp *et al.*, 1972; and Results). Strain W1655 from the laboratory of Broda is λ^R. The original W1655 from the Lederberg laboratory is λ^S (B. Bachmann, personal communication).

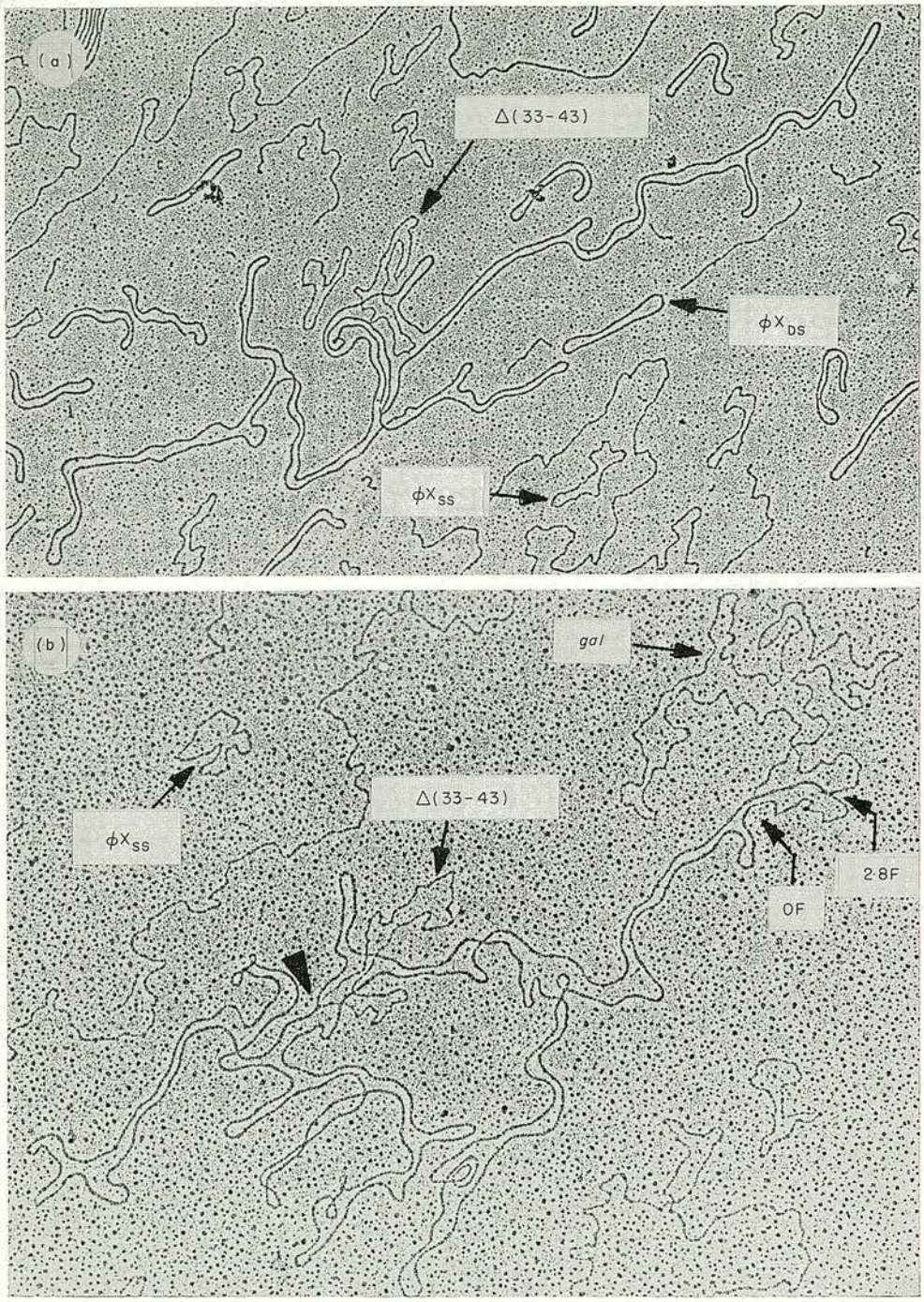

PLATE I (a) FΔ(33-43)/F heteroduplex as depicted in Fig. 1(a). (b) FΔ(33-43)/F152-1 heteroduplex as depicted in Fig. 1(c). Various features of the molecules are marked on the micrographs. φX174 single-stranded and double-stranded molecules are present and were used as length standards. Their length is approximately 5·1 kb.

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deletion. Direct measurements of the single-strand loop indicate that the deletion has a length of 10 ± 1 kb. In the F8-33/FΔ(33-43) heteroduplex, the 33-43 deletion loop is mapped at approximately 17 kb to the right of the point 16·3F, which marks the clockwise terminus of the F8-33/F non-homology substitution loop. The clockwise direction is demonstrated by the position of a little loop, *c*, at 91·0F kb. Thus the deletion has co-ordinates of approximately 33 to 43F kb.

The structure is confirmed by the F152-1/FΔ(33-43) heteroduplex. In this case the 33-43 deletion loop is observed to be 29·7 kb displaced from one terminus of the F152-1/F substitution loop. From this structure alone, one cannot tell whether the deletion is clockwise or counterclockwise relative to the F152-1/F substitution feature, but this ambiguity is resolved by the F8-33/FΔ(33-43) heteroduplex described above.

The co-ordinates of the deleted region can be calculated from measurements of the duplex lengths between the 33-43 deletion loop and the several non-homology features of the reference F-primes. The total length of the deleted region can be obtained by measuring the single-strand length of the deletion loop and, independently, as a difference between the duplex lengths of F and FΔ(33-43). We also take the length of standard F as 94·5 kb. An analysis of all the data on FΔ(33-43) heteroduplexes gives co-ordinates of 32·6–42·9F for the deleted region. Therefore, the calculated value for the length of FΔ(33-43) is $84{\cdot}3 \pm 1{\cdot}0$ kb. This is consistent with the molecular weight ($83{\cdot}8 \pm 1{\cdot}6$ kb) described above.

We observe that *E. coli* W1655 carrying FΔ(33-43) is sensitive to phage τ by the tests described in Materials and Methods. The control tests with the typical male strain, JE3100 carrying an F *gal*, F8-2, and the isogenic F^- strain, JE2571, gave the expected results that the former was not sensitive to phage τ, whereas the latter was.

4. Discussion

FΔ(33-43) is an additional useful reference F for heteroduplex mapping of other F-primes. Its application for these purposes is illustrated in our previous papers (Ohtsubo *et al.*, 1974*a*).

The F-prime factors derived from F100 and F152 are deleted in the F sequences 0 to 2·8F (Ohtsubo & Hsu, 1974). The F8 episomes are deleted in the F sequences 8·5 to 16·3 kb. There is a deletion mutant of F, FΔ(0-15·0) that is deleted in the sequences indicated (previously called FΔ(0-14·5)) (Sharp *et al.*, 1972). The F-prime F210, is deleted in the sequences 8·5 to 11·5F. The plasmid F13-4 is deleted in the F intervals 16·3 to 37·2 kb (S. Hu & E. Ohtsubo, personal communication). The F′*ilv* F16 is deleted in the sequences 8·5 to 35·8F (Lee *et al.*, 1974). All of these F and F-primes are fertile (*tra*$^+$) and capable of autonomous replication, as is FΔ(33-43). Thus, the sequences of F from 0 to 42·9 are not essential for these functions. As discussed previously, the region from 0 to 16·3F seems to be involved in the recombination of F with the *E. coli* chromosome to form Hfr's and in subsequent excisions to give F-primes (Sharp *et al.*, 1972; Ohtsubo & Hsu, personal communication).

Strain W1655 carrying FΔ(33-43) is sensitive to phages T3 (Schell *et al.*, 1963), ϕII (P. Broda, personal communication) and τ as reported here. Many strains carrying F or F-prime factors are resistant to these female-specific phages. Strain 58-161F$^+$ is resistant to T3 (Schell *et al.*, 1963). These observations are consistent with the hypothesis that the gene(s) conferring resistance to female-specific phages lie in the 33-43 region that is deleted in FΔ(33-43).

This research has been supported by the United States Public Health Service by a research grant (no. GM10991) to one of us (N. D.), by support as trainees under GM01262 to other authors (W. M .A.) and (H. J. L.) and by a postdoctoral fellowship to another author (R. C. D.). This is contribution no. 4831 from the Department of Chemistry, California Institute of Technology, Calif., U.S.A.

REFERENCES

Bachmann, B. J. (1972). *Bacteriol. Rev.* **36**, 525–557.
Broda, P. (1967). *Genet. Res. Camb.* **9**, 35–47.
Hakura, A., Otsuji, N. & Hirota, Y. (1964). *J. Gen. Microbiol.* **35**, 69–73.
Lee, H. J., Ohtsubo, E., Deonier, R. C. & Davidson, N. (1973). *J. Mol. Biol.* **89**, 585–597.
Ohtsubo, E., Nishimura, Y. & Hirota, Y. (1970). *Genetics*, **64**, 173–188.
Ohtsubo, E., Deonier, R. C., Lee, H. J. & Davidson, N. (1974*a*). *J. Mol. Biol.* **89**, 565–584.
Ohtsubo, E., Soll, L., Deonier, R. C., Lee, H. J. & Davidson, N. (1974*b*). *J. Mol. Biol.* **89**, 631–646.
Schell, J., Glover, S. W., Stacey, K. A., Broda, P. M. A. & Symonds, N. (1963). *Genet. Res. Camb.* **5**, 483–484.
Sharp, P. A., Hsu, M. T., Ohtsubo, E. & Davidson, N. (1972). *J. Mol. Biol.* **71**, 471–497.

Molec. gen. Genet. 130, 153—163 (1974)

An *Escherichia coli* K12 Mutant Apparently Carrying Two Autonomous F-prime Factors

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Received December 7, 1973

Summary. A multiply auxotrophic RecA$^-$ F$^-$ strain was infected with pairs of F′ factors from RecA$^-$ donor strains. Five pairs of F′ factors gave rise to strains carrying markers originating from both F′ factors. With each strain, one marker but not the other was segregated at low frequency. Both markers were transferred together to a RecA$^-$ recipient, and the marker that could be segregated (but not the other) could also be transferred alone.

Following mutagenesis of the RecA$^-$ F$^-$ strain and its infection with F8 and F30, Gal$^+$His$^+$ progeny were isolated which segregated and transferred Gal$^+$ and His$^+$ independently. An F$^-$ derivative could be reinfected with F8 and F30 to give Gal$^+$His$^+$ "doubles", but, using other F′ factors and pairs of incompatible *fin*$^+$ and *fin*$^-$ R factors, it was not possible to isolate other doubles. Plasmid DNA from such Gal$^+$His$^+$ strains sedimented at 104S, the S-value of both F8 and F30. We suggest that F8 and F30 are both present, but that the breakdown in incompatibility that allows this is very specific.

On long term storage of Gal$^+$His$^+$ clones, His$^+$ is preferentially integrated into the chromosome, so that the ability to transfer His$^+$ is lost and Gal$^-$His$^+$ segregants are F$^-$ and carry no plasmid DNA.

Introduction

Incompatibility between F factors (Scaife and Gross, 1962; Maas and Maas, 1962; Echols, 1963; Maas, 1963; DeHaan and Stouthamer, 1963; Dubnau and Maas, 1968) is of interest as an aspect of the control of plasmid replication. It is also an obstacle to the performance of complementation tests between F factors mutant in replication functions (Cuzin and Jacob, 1967a). It has been reported (Echols, 1962; DeHaan and Stouthamer, 1963; Cuzin and Jacob, 1967b) that strains carrying cohabiting F′ factors can be isolated. However, it is likely (Maas and Goldschmidt, 1969) that the apparent maintenance of autonomous F′ factors is made possible by recombinational interactions between the F′ factors, and between the F′ factors and the chromosome. The report of Palchoudhury and Iyer (1971) of apparent cohabitation of two F′ factors in a temperature-sensitive *dnaB* background is unfortunately concerned with a *rec*$^+$ strain only.

In the work reported here, we have used a number of F′ factors, carrying different markers and in a RecA$^-$ host, and an appropriately marked auxotrophic RecA$^-$ recipient strain. By crosses between these strains, followed by selection, we have been able to isolate strains carrying genes from two different F′ factors, and to characterise these strains by genetic and physical methods. One such strain was chromosomally mutant in that it apparently allowed the cell to harbour two F′ factors simultaneously.

Materials and Methods

Materials. Reagents and enzymes and their sources were as follows: Triton X-100 and deoxyadenosine, Sigma Chemical Co; lysozyme, Worthington, Freehold, N.J.; [methyl-^{3}H] thymidine (20.5 Ci/mmol) and [2-^{14}C] thymidine (62 mCi/mmol), Amersham, England.

Bacterial Strains. The strains used are described in Table 1. F′ factors were maintained in strain ED1221, a *recA* derivative of strain JC6554, an F$^-$ $trp_{am}leu_{am}$ *lac spc* strain obtained from M. Achtman. Strain ED1314 was a *recA*$^-$ *gal*$^-$*met*$^-$*thy*$^-$ strain isolated in several steps from strain JC4566 (obtained from M. Achtman), which has the genotype $trp_{am}leu_{am}$ *his str tsx*. Strain ED1308 was a spontaneous Spc^r derivative of strain ED1314. Strain SA291 was obtained from J. Davison.

Phage Strains and Techniques. The male-specific phage MS2 and the female-specific phage ϕII were provided by N. S. Willetts. Phage stocks were prepared by a confluent plate lysis technique, sterilised with chloroform and stored at 4°C. Such lysates routinely contained 10^{10} to 10^{11} phage/ml. $\lambda pgal_8$ was the gift of J. Davison.

Media. The complex and minimal media used have been described by Willetts and Finnegan (1970).

Mutagenesis. N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was dissolved in *M* citrate buffer (pH 5.5) at a concentration of 0.15 mg/ml and sterilized by filtration. Aliquots of 5 ml of an exponential culture in L-broth were centrifuged, and resuspended in the same volume of fresh L-broth. NTG was added to give a final concentration of 30 μg/ml. After 30 minutes incubation, this mixture was centrifuged and washed twice in M9 buffer and the cells were resuspended in L-broth.

Mating Conditions. Biparental crosses were performed as described in Finnegan and Willetts (1971), using a donor/recipient ratio of 1:10. Matings were mechanically interrupted with a vibratory shaker (Low and Wood, 1965). In triparental crosses a donor:donor:recipient ratio of 5:5:1 was used.

Acridine Orange Curing. About 5×10^2 cells were inoculated into 2 mls L-broth (pH 7.8) with amounts of acridine orange ranging from 2.5 to 25 μg/ml, and incubated in the dark for 16–20 hours, with shaking. Aliquots were spread or streaked on EMB or L plates, and colonies were patched onto L plates, incubated and replicated onto selective media to determine the loss of F′ markers. It was found that acridine orange, using various conditions and repeated attempts, gave no significant enhancement of the spontaneous rate of F′ loss with derivatives of strain ED1314 (see Results).

Isolation of Strains Carrying Markers from Two Donor F′ Strains. Strain ED1314 was mated in triparental crosses with pairs of F′ strains. After 30′, matings were interrupted and dilutions were plated onto streptomycin-supplemented minimal agar selective for either one or both F′ markers. Tests for cross-feeding by plating donor and recipient mixtures without time for contact formation and for reversion and crosses using each donor singly were all performed as controls.

When the initial selection had been for both markers, 20 progeny colonies were streaked on nutrient agar containing streptomycin; 20 single colonies from each such colony were then patched on nutrient agar and, after incubation, replicated onto the original selective medium. Clones giving growth were re-streaked onto nutrient agar and colonies were patched and replicated as before. After two or three cycles, it was possible, with some exceptions (see Results) to isolate clones that segregated each marker only at low frequency (not greater than 1%). When the initial selection had been for only one marker, 100 colonies were patched on the same medium and after incubation were replicated onto the doubly selective medium. Any clone that grew was then purified as above. After purification, the phenotype of the isolated double strains were checked in appropriate selective liquid minimal medium and also on agar plates. The Rec phenotype was confirmed by testing the UV-sensitivity of patched clones (Clark and Margulies, 1965).

Construction of the Plasmid F8($\lambda pgal_8$) F8 was transferred into strain SA291 from strain ED1232. A lawn of strain SA291/F8 was spotted with $\lambda pgal_8$ and incubated overnight at 37°. Survivors from the phage spot were resuspended in buffer and streaked on L plates to give single colonies which were then tested for lysogeny. A purified lysogenic derivative, strain ED3412, was shown to carry the prophage episomally, since it gave zygotic induction on

Table 1. Bacterial strains used

Strain	Genotype[a]	F′ factor carried	Origin of plasmid
ED1221	trp_{am} leu_{am} *lac spc recA* F⁻	—	—
ED1229	ED1221/F*his*⁺	F30	Matney *et al.* (1966)
ED1232	ED1221/F*gal*⁺	F8	Hirota and Sneath (1961)
ED1283	ED1221/F*lac*⁺ *tsx*⁺ *purE*⁺	F13	Hirota and Sneath (1961)
ED1287	ED1221/F*lac*⁺	F42	Adelberg and Burns (1960)
ED1291	ED1221/F*thr*⁺ *leu*⁺	KLF1	Low (1968)
ED1294	ED1221/F*thr*⁺ *leu*⁺ *proA*⁺	KLF4	Low (1968)
ED1314	trp_{am} leu_{am} *his met lac gal thy str recA* F⁻	—	—
ED1308	ED1314 *spc*	—	—
SA291	$(gal\ att\lambda\ bio\ uvrB)_{\Delta}$ *his str*	—	—
ED3412	SA291/F8 ($\lambda pgal_8$)	F8($\lambda pgal_8$)	this paper
ED2763	*trp lac gal*/F*gal*⁺ att_{λ} *bio*⁺ *uvrB*⁺	F100	Low (1973)
JC6535	*his trp lac spc*/F*his*⁺	F57	Willetts and Bastarrachea (1972)
J53(R15)	*pro met*/R15	—	Watanabe *et al.* (1964)
1100(R245)	*thi end*I⁻/R245	—	Bannister (1969)
ED1979	*trp lac*/R100-1	—	Egawa and Hirota (1962)
ED1980	*trp lac*/R1–19	—	Meynell and Datta (1967)

[a] The nomenclature is that recommended by Taylor and Trotter (1972).

transfer to a non-lysogenic recipient. DNA preparations of strain ED3412 contained an homogeneous plasmid species 20S larger than the parental F8 molecule.

Isolation of Plasmid DNA. Cells were grown in M9-glucose minimal medium supplemented with 0.75% (w/v) casamino acids, 20 μg/ml tryptophan and 3 μg/ml thymidine. Labelling was for 2 generations at 37° with [H^3] thymidine (10 μCi/ml) or [^{14}C] thymidine (1 μCi/ml) in the presence of deoxyadenosine (300 μg/ml).

Cleared lysates were prepared by the procedure of Clewell and Helinski (1971) with some modifications. The washed cell pellet was resuspended in a 25% sucrose, 0.05 M tris (hydroxymethyl) aminomethane (Tris, pH 8.0) solution, and spheroplasts were made by the addition of lysozyme and ethylenediamine tetraacetic acid (EDTA). Lysis was accomplished by the addition of a solution containing 2% Triton X-100, 0.05 M Tris (pH 8.0), and 0.0625 M EDTA to an equal volume of spheroplasts. The resulting crude lysate was centrifuged at 27000 g for 15′; the pellet, containing the bulk of the chromosomal DNA, was then discarded and the supernatant (the "cleared lysate") was diluted 1:1 with TES (0.05 M Tris, 0.05 M NaCl, 0.005 M EDTA, pH 8.0). Samples (300 μl) of cleared lysates were centrifuged through 4.8 ml 5% to 20% sucrose gradients containing TES and 0.5 M NaCl at 49000 rpm and 0°C for 55 to 65 minutes (depending on the plasmid) using an SW50.1 rotor in a Spinco model L2–50 centrifuge. In gradients containing DNA labelled with a single isotope ^{32}P-labelled MS2 phage was included as a sedimentation marker. Fractions were collected onto Whatman 3MM paper discs, dried, immersed in 5% (w/v) trichloracetic acid, washed in 80% (v/v) ethanol, dried and counted in a Packard Tri-carb liquid scintillation counter using 0.5% (w/v) butyl PBD (Ciba) in toluene as scintillant. Approximate sedimentation coefficients were calculated with reference to the MS2 marker (81.5 S, Kline and Helinski, 1971) assuming linear sedimentation. Identification of the fast peaks as covalently-closed molecules was confirmed by observing their conversion to the more slowly sedimenting nicked forms on storage.

Results

Isolation of Spontaneously Arising "Double" Strains. Pairs from the first six F′ strains listed in Table 1 were mated in triparental crosses with strain ED1314

(see Materials and Methods). On selection for one marker only, standard-sized progeny appeared within 2–3 days. On selection for both markers small mixed clones appeared after 6–7 days at about 30% of the yield on selection for one marker. Such plates also gave a very few standard-sized colonies after 2–3 days. Some of these were spontaneous Str^R mutants of one or the other donor strain, while others were recipient strains which were revertant for one or the other of the selected markers.

Stable "double" clones were isolated (see Materials and Methods); most were obtained readily, although Gal$^+$His$^+$ "doubles" and "doubles" from crosses with strain ED1283 (carrying F 13) as a parent could not be isolated. All "doubles" were Rec$^-$ and male, since they were sensitive to UV-irradiation and to phage MS2, and resistant to phage ϕII.

Gal$^+$Leu$^+$ "doubles" were isolated using F8 and both KLF1 and KLF4. They segregated Gal$^-$Leu$^+$ clones at low frequency, suggesting that at least Gal$^+$ is carried autonomously, but Gal$^+$Leu$^-$ clones were never obtained. Neither with any of the "doubles" we have studied nor with ED1314 derivatives carrying KLF1 or KLF4 alone could we obtain curing. In crosses between such Gal$^+$Leu$^+$ strains and the RecA$^-$ strain ED1308, only Gal$^+$Leu$^+$ and Gal$^+$Leu$^-$ progeny could be isolated. That is, all clones isolated as Leu$^+$ were also Gal$^+$, although some Gal$^-$Leu$^+$ progeny would have been expected had Leu$^+$ transfer been completely independent of Gal$^+$ transfer. Therefore although both Gal$^+$ and Leu$^+$ are apparently independent of the chromosome for at least part of the time, since they can be transferred to strain ED1308, their relation to each other is unclear.

His$^+$Leu$^+$ and His$^+$Lac$^+$ "doubles", isolated from F30 and KLF1 or KLF4, and F30 and F42, were similar to the Gal$^+$Leu$^+$ "doubles". Each gave His$^-$ segregants at low frequency, but not Leu$^-$ and Lac$^-$ segregants, respectively. They also gave analogous patterns of transfer. With each, both markers were transferred together, and each gave progeny with one marker (His$^+$) alone but not the other (Leu$^+$ or Lac$^+$).

The Effect of Mutagenesis upon the Formation of "Doubles". No Gal$^+$His$^+$ "doubles" were isolated, using the above procedures, when donors carrying F8 and F30 were used. Neither did NTG treatment of one or the other or both donor strains before mating (see Materials and Methods) allow us to isolate such "doubles". However when strain ED1314 was mutagenised and then mated with the F8 and F30 donors Gal$^+$His$^+$ progeny were obtained. The majority segregated Gal$^-$ but not His$^-$ colonies; clones of this type will be discussed in a later section. The remainder segregated both Gal$^-$ and His$^-$ clones at low frequency; among fifteen such clones, eleven transferred only Gal$^+$ to strain ED1308, whereas the others, strains ED2602, ED2604, ED2618 and ED2620, gave both, Gal$^+$ and His$^+$ progeny. When Gal$^+$His$^+$ progeny were selected for, only small colonies were obtained; these proved to be mixed clones of Gal$^+$His$^-$ and Gal$^-$His$^+$ cells. It appears therefore that Gal$^+$ and His$^+$ cannot coexist stably after transfer to strain ED1308. This suggests that the cohabitation observed in ED2602 and its siblings is due to a host function rather than a plasmid function. This was confirmed in reinfection experiments using strain ED3420, an F$^-$Gal$^-$His$^-$ segregant of strain ED2602, and strain ED1314 (Table 2). Strain ED3420 yields

Table 2. Triparental crosses using ED3420 and ED1314 as the reciepient strains

Recipient strain	Progeny/100 recipients		
	Gal$^+$	His$^+$	Gal$^+$ His$^+$
ED3420	7.3	9.4	4.4
ED1314	6.9	14.6	$<10^{-4}$

The donors were strains ED1229 and ED1232. Crosses were performed as described in Materials and Methods.

Table 3. Transfer abilities of "doubles" formed by reinfecting strain ED3420 with F8 and F30

Donor strain	Plasmid(s) carried	Progeny/100 donors	
		Gal$^+$	His$^+$
ED3465	F8, F30	9.2	3.0
ED3466	F8, F30	14	1.1
ED3467	F8, F30	16	1.3
ED3451	F30	—	4.0
ED3471	F8	9.6	—

Biparental crosses were performed as described in Materials and Methods using strain ED1308 as the recipient. The single F′ derivatives, strains ED3451 and ED3471, are included for comparison.

stable Gal$^+$His$^+$ progeny at high frequency, whereas strain ED1314 does not. Most of these Gal$^+$His$^+$ progeny transferred only Gal$^+$, but about a third of them transferred Gal$^+$ and His$^+$ independently, in the sense that progeny selected as carrying one marker usually did not carry the other. Table 3 shows the transfer efficiency of some such clones. The growth rates of the Gal$^+$His$^+$ doubles were similar to that of the parent strain, ED1314, but the colonies had a mucoid morphology.

Plasmid DNA from the Double Strain ED3465. The independent existence of the two plasmid molecules in strain ED3420 was further suggested by an examination of its plasmid DNA. A culture of strain ED3465, a derivative of strain ED3420 able to transfer both Gal$^+$ and His$^+$, was labelled with [^{14}C] thymidine. It was confirmed that at least 99% of the culture consisted of Gal$^+$His$^+$ cells and that it was fertile for both Gal$^+$ and His$^+$. Before lysis the cells were mixed with [^{3}H] thymidine-labelled cells of either strain ED3420 or strain ED3471, which carries F8. Cleared lysates were analysed on neutral sucrose density gradients (Figs. 1B and A respectively). Comparison of the ^{3}H profiles of Fig. 1A and B shows that the fast peak which we observe represents F8 DNA. The plasmid DNA of strain ED3465 co-sediments with F8 DNA; it could therefore represent either a population of F8 and F30 DNA molecules, since F8 and F30 co-sediment (Fig. 1C), or a homogenous population of a single plasmid species. Since no faster-sedimenting structures were observed, it is unlikely that a fused molecule (Willetts and Bastarrachea, 1972) carrying both Gal$^+$ and His$^+$ was present.

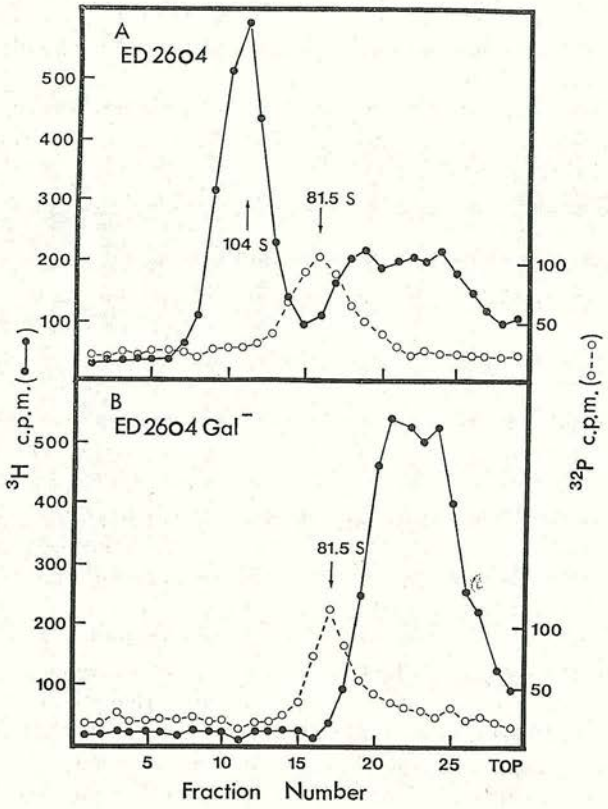

Fig. 2A and B. Neutral sucrose gradient analysis of cleared lysates of strain ED2604 which after storage has lost the ability to transfer His$^+$ (A) and a Gal$^-$His$^+$ segregant of the same strain (B)

among the clones that gave both Gal$^-$ and His$^-$ segregants, eleven among fifteen transferred only Gal$^+$. The most likely hypothesis is that in these cases too His$^+$ has become chromosomally integrated. On only one occasion was a Gal$^+$His$^+$ clone able to transfer His$^+$ but not Gal$^+$ observed.

Attempts to Construct Doubles with Other Plasmid Pairs. Several other F′ factors were tested for their ability to form "doubles" in strain ED3420, with a view to physically resolving the two F′ components of a "double" on sucrose gradients. The pairs tested were KLF1/F57, KLF1/F30, KLF1/F8 and F100/F30. For all pairs the majority of progeny colonies were unstable. Among those which were stable some had lost the ability to transfer one of the markers; in the others the two markers always showed 100% cotransfer and were maintained stably together in the recipient, strain ED1308. Some strains in the last category were shown to carry a new, larger, plasmid DNA species; these were presumably fused derivatives of the separate F′ factors (Palchaudhuri *et al.*, 1972; Willetts and Bastarrachea, 1972).

When the F8 molecule was extended by integration of $\lambda\, pgal_8$ phage DNA into the episome (see Materials and Methods) the new plasmid was unable to form stable "doubles" with F30.

Representatives of two other plasmid incompatibility groups, R100-1/R1–19 (group FII, Hedges and Datta, 1972) and R15/R245 (group N, Datta and Hedges, 1971), also failed to give "doubles".

Discussion

When two incompatible plasmids are introduced into a cell, either one or the other is lost, due to a failure in replication and/or segregation as the cell grows and divides (Dubnau and Maas, 1968; Novick and Brodsky, 1972; Falkow *et al.*, 1971; LeBlanc and Falkow, 1972). The models of Jacob *et al.* (1963) (the "site" model) and of Pritchard *et al.* (1969) (the "repressor" model) have provided the basis for discussion of the mechanism of incompatibility, but it may be that incompatibility is more complex a process than that envisaged in these models. Three examples illustrate this complexity; the first is the demonstration (Novick and Brodsky, 1972) that in *Staphylococcus aureus* the onset of replication and the establishment of a newly introduced plasmid occur at different times. This may also be so in *E. coli* (LeBlanc and Falkow, 1972). Second, F′ factors isolated from an Hfr strain with a mutation in the sex factor, removing the incompatibility barrier against autonomous F′ factors, were themselves subject to incompatibility (DeVries and Maas, 1973). Third, not all F′ factors are equivalent, since (deHaan and Stouthamer, 1963; Echols, 1963) F8 tends to remain as the surviving plasmid when it and another plasmid are introduced simultaneously or when it is either the resident or the incoming plasmid in a superinfection experiment. It may be that F8 is anomalous in some way, since it is unclear whether "Hfr8", its parent strain, was a true Hfr strain (Hirota and Sneath, 1961). Analogous cases of preferential maintenance have been reported with other plasmids (Macfarren and Clowes, 1967; Frydman and Meynell, 1969).

Our main conclusion is that host cells have a role in the expression of incompatibility. We have described a host mutant which, when infected with F8 and F30, gives stable Gal$^+$His$^+$ clones that both segregate (at low frequency) and transfer these markers independently. Moreover, from examination of their plasmid DNA we conclude that in such strains F8 and F30 are present as independent structures, rather than as a fused structure that dissociates during or after transfer. On the basis of experiments with a DnaB$^-$ Rec$^+$ strain Palchoudhury and Iyer (1971) have drawn similar conclusions. Although the strains used were all RecA$^-$, we cannot completely exclude reversible chromosomal integration; however, if this occurred, Hfr/F′ incompatibility (Scaife and Gross, 1962; Dubnau and Maas, 1968) would still be expected to inhibit replication of the autonomous plasmid. The chromosomal integration of His$^+$ on storage by some form of "illegitimate" recombination (Franklin, 1967; Inselburg, 1967; Press *et al.*, 1971; Broda and Meacock, 1971; Willetts and Bastarrachea, 1972) strengthens our conclusion that both plasmids are present as independent replicons in these strains. Their relative stability, although the number of plasmid copies is low, suggests that each plasmid is replicated and segregated equitably. We do

not understand the significance of the fact that this cohabitation is specific for F8 and F30, to the extent that F8($\lambda pgal_8$) cannot substitute for F8.

Non-equivalence of plasmids is a recurrent theme in the work reported here. One case is the specificity shown by our mutant host strain, with respect to F8 and F30. Another is that without mutagenesis we were able to isolate only certain types of "doubles". The nature of these strains is obscure, although they probably arose during the isolation procedure in an unknown number of steps. Once isolated, such "doubles" behaved asymmetrically: if A^+ and B^+ were the F′ markers only A^+B^+ and A^+B^- progeny, and not A^-B^+ progeny, could be obtained in crosses, and only A^-B^+ segregants were observed. It may be significant that both F8 and F30, which together did not give "doubles" of this type, are in this notation class A plasmids.

A final example of non-equivalence is in the preferential integration of His^+ into the chromosome of Gal^+His^+ "doubles" which was observed in the initial isolation, upon storage and reinfection of strain ED3420. This could be a pecularity of the "illegitimate" recombination system responsible, or a consequence of the way in which F8 and F30 interact in our mutant strains. It could also be a function of the inability of F8 to exist readily in a chromosome-integrated state, since a portion of F8 thought to be involved in integration is deleted (Sharp *et al.*, 1972). It is unclear whether these examples of non-equivalence are solely plasmid-determined or whether the host cell also has a role.

Acknowledgement. R.T. was supported by a Medical Research Council scholarship.

References

Adelberg, E. A., Burns, S. N.: Genetic variation in the sex factor of *Escherichia coli*. J. Bact. **79**, 321–330 (1960)

Bannister, D.: Restriction and modification controlled by resistance transfer factors. Ph. D. Thesis, Edinburgh University 1969

Broda, P., Meacock, P.: Isolation and characterization of Hfr strains from a recombination-deficient strain of *Escherichia coli*. Molec. gen. Genet. **113**, 166–173 (1971)

Clark, A. J., Margulies, A. D.: Isolation and characterization of recombination deficient mutants of *Escherichia coli* K12. Proc. nat. Acad. Sci. (Wash.) **53**, 451–459 (1965)

Clewell, D. B., Helinski, D. R.: Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**, 4428–4440 (1971)

Cuzin, F., Jacob, F.: Mutations de l'épisome F d'*Escherichia coli*. II. Mutations à replication thermosensible. Ann. Inst. Pasteur **112**, 397–418 (1967a)

Cuzin, F., Jacob, F.: Association stable de deux épisomes F différents dans un clone d'*Escherichia coli*. Ann. Inst. Pasteur **113**, 145–155 (1967b)

Datta, N., Hedges, R. W.: Compatibility groups among fi⁻ R factors. Nature (Lond.) **234**, 222–223 (1971)

de Haan, P. G., Stouthamer, A. H.: F-prime transfer and multiplication of sexduced cells. Genet. Res. **4**, 30–41 (1963)

DeVries, J. K., Maas, W. K.: Chromosomal integration of F′ factors in recombination-deficient Hfr strains of *Escherichia coli*. J. Bact. **106**, 150–156 (1971)

DeVries, J. K., Maas, W. K.: Description of an incompatibility mutant of *Escherichia coli*. J. Bact. **115**, 213–220 (1973)

Dubnau, E., Maas, W. K.: Inhibition of replication of an F′*lac* episomo in Hfr cells of *Escherichia coli*. J. Bact. **95**, 531–539 (1968)

Echols, H.: Properties of F′ strains of *Escherichia coli* superinfected with F-lactose and F-galactose episomes. J. Bact. **85**, 262–268 (1963)

Egawa, R., Hirota, Y.: Inhibition of fertility by multiple drug-resistance factor in *Escherichia coli* K12. Jap. J. Genet. **37**, 66–69 (1962)

Falkow, S., Tompkins, L. S., Silver, R. P., Guerry, P., LeBlanc, D. J.: The replication of R-factor DNA in *Escherichia coli* K12 following conjugation. Ann. N. Y. Acad. Sci. **182**, 153–171 (1971)

Finnegan, D. J., Willetts, N. S.: Two classes of *Flac* mutants insensitive to transfer inhibition by an F-like F factor. Molec. gen. Genet. **111**, 256–264 (1971)

Franklin, N. C.: Extraordinary recombinational events in *Escherichia coli*. Their independence of the *rec*$^+$ function. Genetics **55**, 699–707 (1967)

Frydman, A., Meynell, E.: Interactions between de-repressed F-like R factors and wild type colicin B factors: superinfection immunity and repressor susceptibility. Genet. Res. **14**, 315–332 (1969)

Hedges, R. W., Datta, N.: R124, an fi$^+$ R factor of a new compatibility class. J. gen. Microbiol. **71**, 403–405 (1972)

Hirota, Y., Sneath, P. H. A.: F′ and F-mediated transduction in *Escherichia coli*. Jap. J. Genet. **36**, 307–318 (1961)

Inselburg, J.: Formation of deletion mutations in recombination-deficient mutants of *Escherichia coli*. J. Bact. **94**, 1266–1267 (1967)

Jacob, F., Brenner, S., Cuzin, F.: On the regulation of DNA replication in bacteria. Cold Spr. Harb. Symp. quant. Biol. **28**, 329–348 (1963)

Kline, B. C., Helinski, D. R.: F1 sex factor of *Escherichia coli*. Size and purification in the form of a strand-specific relaxation complex of supercoiled deoxyribonucleic acid and protein. Biochemistry **10**, 4975–4980 (1971)

LeBlanc, D. J., Falkow, S.: Effects of superinfection immunity on plasmid replication following conjugation. In: Bacterial plasmids and antibiotic resistance (Krčmery, V., Rosival, L., Watanabe, T., eds.), p. 309–318. Prague: Avicenum 1972

Low, B.: Formation of merodiploids in matings with a class of *Rec*$^-$ recipient strains of *Escherichia coli* K12. Proc. nat. Acad. Sci. (Wash.) **60**, 160–167 (1968)

Low, B.: *Escherichia coli* K-12 F-prime factors, old and new. Bact. Rev. **36**, 587–607 (1973)

Low, B., Wood, T. H.: A quick and efficient method for interruption of bacterial conjugation. Genet. Res. **6**, 300–303 (1965)

Maas, R.: Exclusion of an *Flac* episome by an Hfr gene. Proc. nat. Acad. Sci. (Wash.) **50**, 1051–1055 (1963)

Maas, R., Maas, W. K.: Introduction of a gene from *Escherichia coli* B into Hfr and F$^-$ strains of *Escherichia coli* K-12. Proc. nat. Acad. Sci. (Wash.) **48**, 1887–1893 (1962)

Maas, W. K., Goldschmidt, A. D.: A mutant of *Escherichia coli* permitting replication of two F factors. Proc. nat. Acad. Sci. (Wash.) **62**, 873–880 (1969)

Macfarren, A. C., Clowes, R. C.: A comparative study of two F-like colicin factors, ColV2 and ColV3, in *Escherichia coli* K-12. J. Bact. **94**, 365–377 (1967)

Matney, T. S., Goldschmidt, E. P., Erwin, N. S., Scroggs, R. A.: A preliminary map of genomic sites for F-attachment in *Escherichia coli* K12. Biochem. biophys. Res. Commun. **17**, 278–281 (1964)

Meynell, E., Datta, N.: Mutant drug resistance factors of high transmissibility. Nature (Lond.) **214**, 885–887 (1967)

Novick, R. P., Brodsky, R.: Studies on plasmid replication. I. Plasmid incompatibility and establishment in *Staphylococcus aureus*. J. molec. Biol. **68**, 285–302 (1972)

Palchaudhuri, S. R., Mazaitis, A. J., Maas, W. K., Kleinschmidt, A. K.: Characterisation by electron microscopy of fused F-prime factors in *Escherichia coli*. Proc. nat. Acad. Sci. (Wash.) **69**, 1873–1876 (1972)

Palchoudhury, S. R., Iyer, V. N.: Compatibility between two F′ factors in an *Escherichia coli* strain bearing a chromosomal mutation affecting DNA synthesis. J. molec. Biol. **57**, 319–333 (1971)

Press, R., Glansdorff, N., Miner, P., DeVries, J., Kadner, R., Maas, W. K.: Isolation of transducing particles of $\phi 80$ bacteriophage that carry different regions of the *Escherichia coli* genome. Proc. nat. Acad. Sci. (Wash.) **68**, 795–798 (1971)

Pritchard, R. H., Barth, P. T., Collins, J.: Control of DNA synthesis in bacteria. Symp. Soc. gen. Microbiol. **19**, 263–296 (1969)

Scaife, J., Gross, J. D.: Inhibition of multiplication of an F-*lac* factor in Hfr cells of *Escherichia coli* K12. Biochem. biophys. Res. Commun. **7**, 403–407 (1962)

Sharp, P. A., Hsu, M., Ohtsubo, E., Davidson, N.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. I. Structure of F-prime factors. J. molec. Biol. **71**, 471–497 (1972)

Taylor, A. L., Trotter, C. D.: Linkage map of *Escherichia coli* K12. Bact. Rev. **36**, 504–524 (1972)

Watanabe, T., Nishida, H., Ogata, C., Arai, T., Sato, S.: Episome mediated transfer of drug resistance in *Enterobacteriaceae*: VII. Two types of naturally occurring R factors. J. Bact. **88**, 716–726 (1964)

Willetts, N. S., Bastarrachea, F.: The genetic and physico-chemical characterization of *E. coli* strains carrying fused F-primes derived from KLF1 and F57. Proc. nat. Acad. Sci. (Wash.) **69**, 1481–1485 (1972)

Willetts, N. S., Finnegan, D. J.: Characterisation of *E. coli* K12 strains carrying both an F-prime and an R factor. Genet. Res. **16**, 113–122 (1970)

Communicated by W. Maas

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Rate of segregation due to plasmid incompatibility

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(*Received* 18 *August*, 1978)

SUMMARY

We calculated the rates of segregation due to plasmid incompatibility under several simple models. A common feature of all the models that we considered is that incompatibility is caused by the inability of the segregation mechanism to distinguish between two incompatible plasmids.

We measured the rate of segregation due to incompatibility of a pair of ColE1 derivatives under two conditions: (1) One plasmid was introduced into cells carrying the other by conjugation. (2) Cells carrying both plasmids were maintained by selection and then selection was released.

Interpretation of the results was made more difficult by effects of the plasmids on the host cell's growth rate. These experiments gave results in agreement with the predictions of a random pool replication model. Published results were also in reasonable agreement with this model.

1. INTRODUCTION

Two closely related plasmids are usually unable to coexist in the same cell, a perty called 'incompatibility'. San Blas, Thompson & Broda (1974) and De es *et al.* (1975) reported the maintenance of two F′ plasmids in the same cell. wever, this was very specific for the particular pairs of F′ plasmids and no eral incompatibility mutations for autonomous plasmids have been isolated. e lack of success in isolating incompatibility mutations makes it attractive to sider models in which incompatibility is an inevitable by-product of normal lication and segregation functions.

Jacob, Brenner & Cuzin (1963) suggested a model for replication and segregation which there exist membrane sites which are responsible for the replication of a smid and these membrane sites divide at cell division and segregate one copy the replicated plasmid to each daughter cell. Incompatibility would be due to mpetition for a limited number of membrane sites. If plasmids occupied the mbrane sites throughout the cell cycle then an incoming incompatible plasmid uld be unable to replicate because the membrane sites would all be occupied by

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the resident plasmid. However, two incompatible plasmids can frequently b established in a cell by selection and, on release of selection, segregation is ofte fairly symmetrical between the two plasmids (Echols, 1963; Uhlin & Nordström 1975; Cabello, Timmis & Cohen, 1976; Timmis, Andres & Slocombe, 1978). Th suggests that the attachment to any site is not permanent although recombinatio between plasmids might account for some of the apparent displacement of th resident plasmid. If there is only one site per cell then if a cell contains two incom patible plasmids (one copy of each) one will win the competition for the site an the other will not be replicated and enter only one of the daughter cells. Therefor the proportion of cells carrying both plasmids will fall by half each generatio This is much faster segregation than that observed even for the low copy numbe plasmids R1 (Uhlin & Nordström, 1975) and F′ plasmids (Jamieson & Bergquis 1977). If there were more than one site per cell then two plasmids with th same replication and segregation system should be able to coexist in the sam cell.

The essential feature of the model of Jacob, Brenner & Cuzin (1963) is the tigh coupling between replication and segregation. Because of the failure of suc models, we consider models in which replication and segregation are independen Such independence has been suggested for *Staphylococcus aureus* plasmids (Novic & Schwesinger, 1976).

The model of Jacob *et al.* (1963) also predicts a 'democratic' mode of replic tion, i.e. every plasmid copy is replicated once per generation. This can be teste by using a density shift experiment. The democratic model predicts that twic replicated plasmid DNA should not appear until one generation after the shif However, when experiments were done using the plasmids NR1 (Rownd, 1969 ColE1 (Bazaral & Helinski, 1970), R1 (Gustafsson & Nordström, 1975), and I plasmids (Kline, 1974; Gustafsson, Nordström & Perram, 1978) twice-replicate DNA appeared much more quickly and the results were in better agreement wit the random pool model of replication. Kline (1974) interpreted his results as due a democratic replication model with the twice-replicated DNA being produce as a result of a disturbance of replication due to his bromouracil density labe Finkelstein & Helmstetter (1977) suggested that the results of Gustafsson *et a* (1978) were due to a disturbance in cell growth caused by the density shif However, the simplest interpretation of these results is that the democrat replication model is invalid. The data are consistent with the random pool mod but do not prove that this model is valid. In this paper we mainly consider th random pool model. We assumed that replication control acted to restore th number of plasmid copies per cell to a constant number before division.

Positive control of plasmid replication is predicted by the model of Jaco Brenner & Cuzin (1963). Pritchard, Barth & Collins (1969) put forward a alternative negative control model for plasmid replication. Cabello *et al.* (197 tested the predictions of these models by linking together two compatib plasmids of different copy number. The Jacob model predicts that such a plasm should have a copy number equal to the sum of the copy numbers of the co

tuent plasmids and replication should occur from both origins. In contrast, the itchard model predicts that the plasmid should have a copy number equal to at of its higher copy number constituent and replicate only from the origin of e higher copy number constituent. The data agreed with the predictions of the ter model.

Two main models for segregation have been proposed (see Novick, Wyman, uanchaud & Murphy, 1975):

(i) Equal-number segregation: an equal number of plasmid copies are distributed each daughter cell.

(ii) Random segregation: plasmids can enter either daughter cell at random on l division. This model predicts that if there are k plasmids in a cell at division en a proportion $1/2^k$ of daughter cells will lack the plasmid. Thus, a plasmid will ly be stable on this model if the copy number is reasonably high.

Novick *et al.* (1975) tested between these models by studying the segregation plasmid-free cells when plasmid replication was blocked. The random gregation model predicts an earlier appearance of plasmid-free cells than the ual number model does under the assumption that equal number segregation ntinues after plasmid replication is blocked. A low copy number plasmid ve results in agreement with the equal number model. Hashimoto-Gotoh Sekiguchi (1977) draw similar conclusions from experiments with pSC101, ich had 10–14 copies per cell. Novick *et al.* (1975) also interpreted the ults of May, Houghton & Perret (1964) with a high copy number plasmid out 32 per cell) as supporting equal number segregation. However, in this case out 20 % of the cells were plasmid-free at the start of the experiment so that it uld have been difficult to detect early new segregants.

The equal number and random segregation models in a sense are extreme cases. r the low copy number plasmids some mechanism must exist to ensure that each ughter cell receives at least one plasmid copy. Another possible model to plain this is that a mechanism ensures the distribution of one copy to each ughter cell and the other copies are distributed at random. Such a mechanism is sier to imagine for higher copy number plasmids than an equal number mechan- n that must pair up many plasmid copies and distribute them to daughter cells. is would be very difficult to distinguish experimentally from the equal number del. However, we found that the choice of segregation model seemed to be atively unimportant in predicting the rates of segregation due to incompati- ity. We did most of our work with an equal number segregation model as the culations are easier than for a random segregation model. We compared the dictions with our own data and with published data. The effects of using other nple models instead were also investigated.

2. MATERIALS AND METHODS

Strains and Media

Bacterial strains are shown in Table 1. Media, growth conditions and phage T6 parations were as described in Cullum, Collins & Broda (1978). Kanamycin

Table 1. *Bacterial strains*

Strain	Description	Source*
ED2510	ED6256 (F'*lac traΔI337*)(pML2)	P1 transduction of pML2 from ED2521 to ED3887
GW137	ED6256 (F'*lac traI65*)(pDS1107)	Dr J. Watson
ED3826	JC3272 derivative resistant to colicin E1	Dr N. Willetts
ED2516	ED3826 (pML2)	ED2510 × ED3826
ED2517	ED3826 (pDS1107)	GW137 × ED3826
ED3887	JC6256 (F'*lac traΔI337*)(ColE1)	Dr N. Willetts
ED2521	C600 (pML2)	Dr D. J. Finnegan

* JC6256 and JC3272 are described in Achtman, Willetts & Clark (1971).

(Winthrop) and ampicillin (Beechams) were added to media at 50 μg/ml whe required.

(ii) *Segregation rate measurements*

(*a*) Equal volumes of exponentially growing cultures at a concentration of abou 5×10^7 cells/ml of ED2510 and ED2517 in one case and GW137 and ED2516 i the other case were mixed. After 40 min mating phage T6 was added to give a fina concentration of about 10^{10} pfu/ml. The first samples were taken 10 min later (tim zero). The cultures were maintained between 2×10^7 and 4×10^8 cells/ml by seria dilution. At intervals, dilutions of samples from the cultures were plated on L broth agar containing either streptomycin or streptomycin, ampicillin an kanamycin. Samples were also plated on lactose tetrazolium agar containin kanamycin (for the ED2510 mating) or ampicillin (for the GW137 mating) t check that the number of Lac$^+$ donor cells surviving phage T6 was less than 1 % of the progeny number.

(*b*) Cells containing both pML2 and pDS1107 were obtained by mating ED251 with ED2517 and plating samples on L-broth agar containing streptomycin kanamycin and ampicillin. After overnight incubation, colonies were streaked ou onto the same medium. Samples from a purified colony were inoculated into 10 m of L-broth in a side arm flask which contained ampicillin and kanamycin. Whe this culture reached about 2×10^8 cells/ml it was spun down in a bench centrifug and resuspended in warm L-broth. The zero time was this time of resuspensio The culture was maintained between 2×10^7 cells/ml and 4×10^8 cells/ml by seria dilution. At intervals dilutions of samples from the culture were plated on strepto mycin-containing L-broth agar which also contained ampicillin or kanamycin c both or neither.

3. RESULTS

(*a*) *Random pool replication models*

The main model that we considered contained the following assumptions:

(i) The cell population is large enough for random fluctuations to be negligibl

(ii) Control of replication is such that all plasmid-carrying cells at division hav

e same number of plasmid copies ($2N$); thus N is the average copy number at
rth.

(iii) Replication and segregation are independent.

(iv) The replication and segregation mechanisms cannot distinguish between a
ir of incompatible plasmids.

(v) Replication follows a random pool model.

(vi) Segregation follows an equal number model.

(vii) The plasmids do not affect the host cell's growth rate.

The first three assumptions are common to all the models that we consider.
owever, later we consider the effects of changing each of the last four assumptions.
e only considered values of N greater than 1 because, if $N = 1$, all cells carrying
o incompatible plasmids would segregate the two plasmids completely at the
xt division.

The initial rate of segregation in a population carrying two incompatible
asmids would depend on the distribution of the two plasmids between cells
rrying both. However, after a few generations the distribution of the two
asmids would settle down to a limiting distribution with a corresponding
nstant steady state rate of segregation, which depends only on the copy number.
is was confirmed by computer modelling. This rate is most easily expressed in
rms of the half-time for segregation, i.e. the number of generations needed for the
oportion of cells carrying both plasmids to fall by half. We calculated the half-
me to be (see Appendix):

$$t_{\frac{1}{2}} = \log_e 2/\log_e ((N+1)(2N-1)/((N-1)(2N+1))), \qquad (1)$$

here N is the copy number at birth. This is approximately equal to $N.\log_e 2$ for
rge values of N (say N greater than 10).

This steady state rate should be appropriate for experiments in which the rate
segregation is observed after both plasmids have been established in the same
lls by selection. However, in nature the initial conditions are likely to be of one
asmid at a numerical disadvantage after entry of one copy into a cell in which the
her plasmid is resident. The higher the copy number the greater this disadvantage
ll be. We modelled this situation by taking initial conditions in which there was
copy of one plasmid and $N-1$ of the other in cells at birth. For $N = 2$ (Fig. 1)
e proportion of cells carrying both plasmids decreases exponentially; in fact, this
just the steady state case discussed earlier with one copy of each plasmid
esent. For higher copy numbers there is an initial high rate of segregation before
e rate settles down to the steady state rate (Fig. 1). Such behaviour was seen by
ovick & Brodsky (1972) after transfer of a plasmid into cells carrying an incom-
tible plasmid by transduction. It is interesting to note that the initial rate of
gregation is almost independent of copy number for copy numbers above about
. The way in which we can use such calculations to estimate copy number from
gregation data will be discussed later.

In order for the calculations to be useful experimentally we have to relate the
py number at birth (N) to the average copy number in exponentially growing

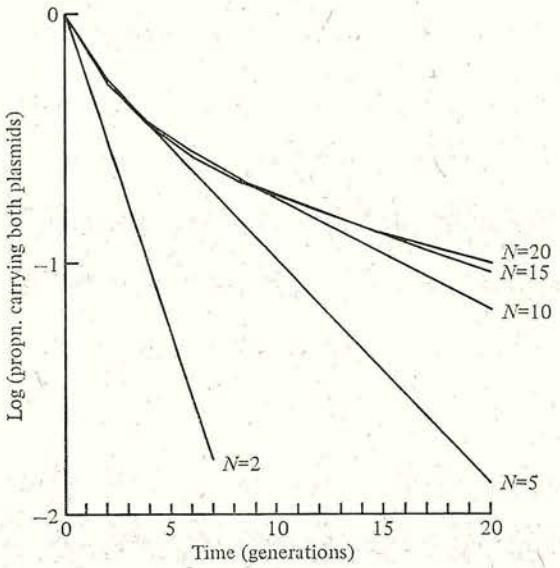

Fig. 1. Proportion of cells that would carry both plasmids, on the model of random pool replication and equal number segregation, starting from newborn cells carrying 1 copy of plasmid 1 and $N-1$ copies of plasmid 2. The model's predictions are shown for $N = 2, 5, 10, 15, 20$. The curves for $N = 30$ and $N = 40$ are too close to that for $N = 20$ to be shown here.

populations ($\bar{N}$). The relationship between N and $\bar{N}$ depends on the timing c plasmid replication in the cell cycle. The system in which this question has bee most studied is that of F′*lac* in *Escherichia coli* B/r. Several groups obtaine results which suggested that F′*lac* replicated at a particular time, though there wa disagreement about the location of this time in the cell cycle (see Finkelstein & Helmstetter, 1977). However, using more direct methods two groups have recentl obtained results that suggested that F′*lac* replication is spread throughout the ce cycle (Gustafsson *et al.* 1978; Andresdottir & Masters, 1978). In the case of th plasmids prophage P1 (Abe, 1974) and R1 (Gustafsson *et al.* 1978) replication ma also be spread throughout the cell cycle.

If replication is spread uniformly through the cell cycle, the problem of relatin $\bar{N}$ to N is analogous to that of relating average cell length ($\bar{L}$) to cell length at birt (L) that was considered by Donachie, Begg & Vicente (1976). They gave th equation $\bar{L} = L/\log_e 2$ and we used the analogous equation for copy numbe $\bar{N} = 1{\cdot}44N$. This will be a reasonable approximation if replication is sprea throughout the cell cycle, whatever the exact dependence. This equation migh still be reasonable even if replication occurs at a particular point in the cell cycl provided it is not too close to cell birth or cell division.

We also considered a random segregation model (i.e. changed assumption (vi where each of the $2N$ plasmid copies in a dividing cell has an equal opportunity o entering either daughter cell. This model produces a proportion $(\frac{1}{2})^{2N}$ plasmid-fre cells per generation; it would therefore be too unstable to apply to lower cop

umber plasmids. We calculated the steady state segregation rate using a compu-
r (see Appendix) and found that the half-time was approximately $0{\cdot}692N - 0{\cdot}67$
the range for N of 5–20. This would, in practice, be indistinguishable from
e rate predicted by the equal number segregation model. The rate of segregation
r the case of one plasmid entering a cell containing the other would also be
distinguishable from that predicted by the equal number model.

Experiments with ColE1 derivatives

We measured the rate of segregation due to incompatibility using a pair of
lE1 derivatives, pML2 (= Kan^R) and pDS1107 (= Amp^R). These could be
obilized by an F'*lac traI* plasmid that was unable to transfer itself (Alfaro &
illetts, 1972). We were thus able to study incompatibility after conjugation in
e absence of retransfer by the recipients as the ColE1 derivatives cannot transfer
emselves. Continuing transfer by the original donors could be eliminated by
ling them with phage T6.

Figure 2 (○ and △) shows how the proportion of cells carrying both plasmids
clines after one is introduced into cells carrying the other by conjugation. There
an initial rapid rate of loss followed by a lower rate of loss. The experimental
ta are in excellent agreement with a theoretical curve based on the assumption
at one copy of the incoming plasmid enters cells containing the other (Fig. 2).
e theoretical curve given is for $N = 20$, but the curves for N in the range 20–40
e indistinguishable (see legend to Fig. 1).

We also measured the steady state segregation rate. We selected cells carrying
th plasmids by growth on medium containing both kanamycin and ampicillin.
e then followed the progress of segregation after selection was removed; Fig. 2
ows such an experiment. We found that even after growth in selective medium
–70 % of cells did not carry both plasmids, as judged by the lower viable count
kanamycin ampicillin agar (KA) than on non-selective agar. This was probably
e to the destruction of the ampicillin in the medium by β-lactamase, as the
jority of these segregants carried only pML2 as the viable counts were indis-
guishable between kanamycin agar (K) and non-selective agar, and between
picillin agar (A) and KA. However, after growth in non-selective medium the
ble counts on K became less than those on non-selective agar and those on KA
came less than those on A which indicated that segregation of cells carrying only
S1107 had occurred. Thus, it seemed that the large initial asymmetry between
e two types of segregants was due to the properties of the antibiotic resistances
ried rather than properties of the segregation mechanism.

As segregation was very slow (Fig. 2, ×) any differential effects of the two
smids on the host growth rate could be important. Both ED2516 (which
ries pML2) and ED2517 (which carries pDS1107) had a generation time of about
min in our growth conditions. We measured the differential growth rate of the
o strains in a mixed culture; samples were plated on non-selective agar to find the
portion of each strain in the mixture at different times. The generation time of
2516 was about 5% less than that of ED2517. A difference of this magnitude

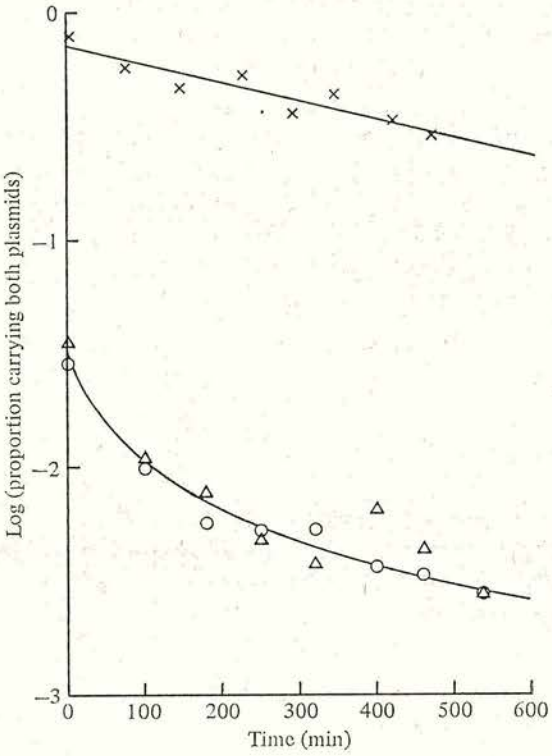

Fig. 2. Ratio of $Kan^R Amp^R$ cells (i.e. cells carrying both plasmids pML2 and pDS1107) to total cell numbers. The generation time was about 22 min. (i) From an ED2510 × ED2517 mating, ○; (ii) from a GW137 × ED2516 mating ($\times 10^2$), △; (iii) starting from a culture grown up in the presence of kanamycin and ampicillin, ×. All $Kan^R Amp^R$ colonies still segregated $Kan^S Amp^R$ and $Kan^R Amp^S$ cells, showing that any stable recombinants could be neglected. The GW137 donor was less fertile than the ED2510 donor in matings with the non-coliginogenic recipient strain ED3826. The theoretical curve for (i) and (ii) above is based on the model used for Figure 1 with $N = 20$. It was fitted to the data using the number of cells containing both plasmids at time 0.

may have an appreciable effect on the estimate of the half-time for segregation We used the results of three segregation experiments to calculate the copy numbe under three different assumptions about differential growth (see Appendix):

(i) If all cells had the same generation time, the average copy number ($\bar{N}$) would be 37·5 copies/cell.

(ii) If cells carrying both plasmids had the same growth rate as ED2517, th average copy number ($\bar{N}$) would be 79·7 copies/cell.

(iii) If cells carrying both plasmids had the same growth rate as ED2516, th average copy number ($\bar{N}$) would be 35·7 copies/cell.

Thus any reduction in growth rate of cells carrying both plasmids will have large effect on the copy number estimate. The higher estimate (assumption (ii) is comparable to the value of 18 covalently closed circular DNA molecules pe

enome equivalent found by Cabello *et al.* (1976) if it is assumed that our cells ontained about 4 genome equivalents of DNA (Cooper & Helmstetter, 1968).

The yields of segregants carrying each type of plasmid can also be predicted nder each of the assumptions made above. The predictions were consistent with ne experimental data. However, as the experimental estimates involve measuring ne differences between viable counts on different selective agar, the data were not ood enough to distinguish the different assumptions about the growth of cells arrying both plasmids. In practice, the growth rate may depend on the number of opies of each plasmid present.

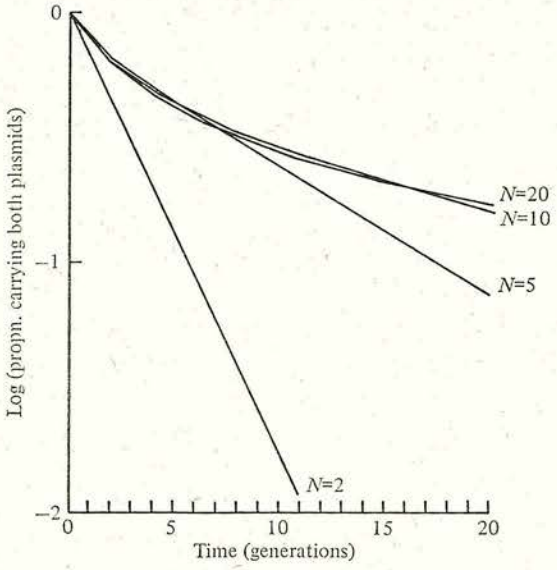

Fig. 3. Proportion of cells that would carry both plasmids, on the model of democratic replication and equal number segregation, starting from newborn cells carrying 1 copy of plasmid 1 and $N-1$ copies of plasmid 2. The model's predictions are shown for $N = 2, 5, 10, 20$. The curve for $N = 15$ is too close to that for $N = 20$ to be shown here.

) *Other replication models*

We also calculated rates of segregation under a democratic replication model. Ve used an equal number segregation model as a random segregation model leads rapid plasmid loss; this is because there is no compensating mechanism to store copy number after unequal divisions. This corresponds to equation (1) of owman (1973) with $p_2 = 1$. We used a computer (see Appendix) to calculate the eady state rate of segregation. We found that the half time was approximately

$$t_{\frac{1}{2}} = 1{\cdot}37N - 0{\cdot}96 \quad (N \text{ in the range } 2\text{–}20). \tag{2}$$

his segregation rate is about half that predicted by random pool models with the me copy number at birth. Figure 3 shows that the rate of segregation when one asmid enters cells containing the other plasmid is also less than in the random ool case (Fig. 1).

In all the models we have discussed so far the two plasmids are indistinguishable to the replication mechanism. However, one plasmid might have an advantage over the other in selection for replication; e.g. there might be differences in the sites on the two plasmids that are recognized by replication-control proteins. We modelled such a case using a random pool replication model in which the two plasmids had an unequal chance of replication. This asymmetry caused an asymmetry in the number of segregants of each plasmid type. We calculated the proportion of final segregants of each type when the cells started with an equal number of copies of each type (see Appendix). Table 2 shows that there is appreciable asymmetry of segregation if the replication probabilities of the two plasmids are in the ratio of 1·1:1; larger differences result in nearly all segregants carrying only the more successful plasmid.

Table 2. *Effect of biased replication on asymmetric segregation*

Copy number at birth (N)	Ratio of probabilities of replication for the two plasmids	Percentage of segregants carrying only	
		(i) More successful plasmid	(ii) Less successful plasmid
10	1·01:1	52	48
10	1·1:1	66	34
10	1·5:1	94	6
10	2:1	99	1
15	1·01:1	53	47
15	1·1:1	72	28

4. DISCUSSION

The model with random pool replication and equal number segregation is arguably the simplest model for incompatibility which is not inconsistent with published data. We, therefore, calculated segregation rates for this model and compared them with experimental data. The model gave good agreement for the case of one ColE1 derivative being transferred into a cell in which another derivative was present (Fig. 2). As the segregation is relatively rapid any differential effect of the plasmids on the host growth rate is relatively unimportant. We also considered the steady state segregation rate. If we assumed that cells containing both plasmids grew at the same rate as cells containing only pDS1107, i.e. 5% slower than cells carrying only pML2, then our estimate (80 copies/cell) is comparable to an estimate (18 copies/genome equivalent) based on the number of covalently closed circular DNA molecules (Cabello *et al.* 1976). However it is unclear what growth conditions they used and under certain circumstances ColE1 copy number can increase considerably (Bazaral & Helinski, 1970). If the effect on growth rate on cells carrying both plasmids is less, the copy number estimate is lower.

It is interesting to note that considerable segregation due to incompatibility occurs even on kanamycin ampicillin medium and that in our case most segregants had lost pDS1107. Study of segregation in non-selective medium showed that this

s not due to asymmetry in segregation; it was probably due to the destruction ampicillin removing selection for pDS1107. This means that observation of ymmetry between segregant types on selective medium does not necessarily lect asymmetry of the incompatibility function. This in turn leads to some ubt about the interpretation of the asymmetry between plasmids observed by nmis *et al.* (1978) when investigating the incompatibility properties of DNA gments cloned from R6-5.

Table 3. *Calculation of copy numbers from published segregation data*

Plasmid	$t_{\frac{1}{2}}$	Calculated $\bar{N}$	Calculated copy number per genome equivalent[a]	Measured copy number per genome equivalent (by CCC DNA unless otherwise indicated)
F	1·8[b]	3·7	1·8	1·2[c] (by hybridization)
pSC101	6·7[d]	13·9	3·5	5[d] 3–5·5[e] (segregation by temperature-sensitive mutants)
ColE1	14[d]	29·2	7·5	18[d]
R1	4·0[f]	8·3	2	0·4[g]
R483	6·9[h]	14·5	3·6	1[i]

This used the relation between DNA content and growth rate of Cooper & Helmstetter 68).
Jamieson & Bergquist (1977).
Collins & Pritchard (1973).
Cabello, Timmis & Cohen (1976).
Hashimoto-Gotoh & Sekiguchi (1977).
Uhlin & Nordström (1975).
Engberg, Hjalmarsson & Nordström (1975).
Datta & Barth (1976).
Barth, Datta, Hedges & Grinter (1976).

t is possible to compare the predictions of the model with published data for y number and segregation rates. There is the problem that segregation rate asurements and copy number measurements are often made under different wth conditions and growth conditions can affect plasmid copy number. This been shown for ColE1 (Bazaral & Helinski, 1970), F′*lac* (Collins & Pritchard, 3) and R1 (Engberg, Hjalmarsson & Nordström, 1975). In the case of two smids (F and pSC101), where copy number has been determined by methods ependent of extracting covalently closed circular DNA, the agreement with our imate based on published segregation rates is fairly good (Table 3). Cabello *l.* (1976) gave data for ColE1 derivatives that gave a half time for segregation ble 3) comparable with our measurements (18 generations). The points we cussed above with respect to our results also apply in this case. For the large smids R1 and R483 the segregation rates gave us copy number estimates eral times those made by measuring the amount of covalently closed circular

DNA (Table 3). At least part of this discrepancy can be explained by the efficienc of recovery of plasmid in the form of covalently closed circles. Thus, the predi tions of our model are not inconsistent with the published data and yield the be results with F and pSC101, which are the cases where the published data probab give the most accurate estimates of copy number.

The appropriate segregation model for high copy number plasmids is uncle (Novick *et al.* 1975). We therefore also considered the random segregation mod and calculated the segregation rates for a random pool replication, rando segregation model. This gave predictions very similar to those with equal numb segregation. Thus, in practice, the rate of segregation of incompatible plasmi could not be used to distinguish the two models. It seems unlikely that suc experiments could be used to choose between any segregation models which do n distinguish the two plasmids. However, the segregation rate could be used distinguish random pool and democratic replication models. A democratic replic tion model gave segregation rates considerably less than those given by a rando pool model of corresponding copy number (cf. equations (1) and (2) and Figs. and 3). The latter model gave a better fit to our experimental data (Fig. 2, ○ an △). Measurements of segregation rate might prove useful in testing betwee random pool models and any other replication models that might be put forwar

One use of our calculations is to predict the copy number of a plasmid from th segregation rate due to incompatibility. If it is assumed that a random po replication model is valid then the steady state rate of segregation will allow th calculation of copy number. However, differential growth rates may affect th estimate; this will be more serious for high copy number plasmids where the ra of segregation is very low. This method of estimating copy number gives estimat of copy number under normal conditions. This is in contrast with methods base on the kinetics of segregation of plasmid-free cells by temperature-sensiti replication mutants at the restrictive temperature, in which the mutations ma affect the copy number at the permissive temperature. The results of the latt experiments are even more difficult to interpret when the mutations are 'leaky i.e. some replication occurs at the restrictive temperature. Then the segregatio rate at later times, when most plasmid-carrying cells have only one plasmid cop allows the estimation of the amount of replication remaining and this must extrapolated back to the start of the experiment to obtain the initial copy numbe However, in principle, there are at least two different assumptions that could made in extrapolating back:

(i) The amount of remaining replication per cell is constant and does not depe on the number of plasmid molecules present in the cell.

(ii) The amount of remaining replication per cell is proportional to the numb of plasmid copies carried.

The methods used so far have made the second assumption. This gave resul in agreement with covalently closed circular DNA measurements for pSC1 (Hashimoto-Gotoh & Sekiguchi, 1977). Durkacz & Sherratt (1973) made th same assumption when considering ColE1 segregation from a temperatur

sitive-*polA* strain; if the first assumption were more appropriate for this case ere a chromosomal mutation is used, then their estimate would be increased 4 times.

Experiments where one plasmid is introduced into cells carrying the other and segregation rate measured are not suitable for finding the copy number of her copy number plasmids. Figs. 1 and 3 show that if the copy number at birth) is greater than 10 there is little variation with copy number over twenty nerations.

In some cases segregation due to incompatibility is asymmetric with one plasmid ng favoured (Macfarren & Clowes, 1967). The symmetric models we have dis-ssed can be adapted in at least two ways to account for this:

i) The probability of replication in the random pool model may not be the same two plasmids; this could be due to differences in the plasmid sites recognized by replication system. Table 2 shows that appreciable asymmetry occurs for copy mbers over 10 if there is a greater than 1·1:1 replication advantage.

ii) One plasmid might carry genes that repress replication of the other plasmid. both of these cases mutations or perhaps even changes in the growth conditions uld be capable of modifying the asymmetry. Changes in growth conditions can olish asymmetry in the case of R483 (Datta & Barth, 1976). In the second case would, in principle, be possible for one plasmid to be incompatible with another t has an unrelated replication system; mutations should then allow both smids to coexist in a cell. In fact, *inc* mutations in Hfr strains which allow the intenance of autonomous F′ plasmids (De Vries & Maas, 1973) can be viewed as tations abolishing incompatibility between the replicons of the chromosome d the autonomous F.

Since this paper was submitted for publication, two further papers in which dels for replication and assortment have been considered have appeared ovick & Hoppensteadt, 1978; Ishii, Hashimoto-Gotoh & Matsubara, 1978).

APPENDIX

Random pool replication, equal number segregation model

f there are s copies of plasmid 1 and t copies of plasmid 2 in a cell then the bability that plasmid 1 is replicated next is $s/(s+t)$ and the probability that smid 2 is replicated next is $t/(s+t)$. If a cell has s copies of plasmid 1 and t of smid 2 then the probability that plasmid 1 is replicated $k-s$ times to give k ies and plasmid 2 is then replicated to give $2N-k$ copies is

$$\left(\frac{s}{s+t}\cdots\frac{k-1}{k+t-1}\right)\left(\frac{t}{k+t}\cdots\frac{2N-k-1}{2N-1}\right)=\frac{(k-1)!\,(2N-k-1)!\,(s+t-1)!}{(s-1)!\,(t-1)!\,(2N-1)!}.$$

wever, the probability of going from s to k copies of plasmid 1 and from t to $-k$ copies of plasmid 2 is independent of the order of replication of the copies of two plasmids. As there are

$$\binom{2N-s-t}{k-s}$$

possible orders, the total probability of going from s to k and from t to $2N-$ copies is

$$P(s \to k, t \to 2N-k) = \frac{\binom{k-1}{s-1}\binom{2N-k-1}{t-1}}{\binom{2N-1}{s+t-1}} \quad \text{for} \quad \begin{cases} 1 \leqslant s \leqslant k \\ 1 \leqslant t \leqslant 2N-k. \end{cases} \tag{A 1}$$

If there is equal number segregation then the probability that there are s copie of plasmid 1 (hence $N-s$ of plasmid 2) in a new-born cell, given that the paren cell had k and $2N-k$ copies of plasmids 1 and 2 respectively at division, is (usin Whittle (1970), equation 4.5.1)

$$P(s|k) = \frac{\binom{k}{s}\binom{2N-k}{N-s}}{\binom{2N}{N}} \tag{A 2}$$

for $$\max(0, k-N) \leqslant s \leqslant \min(N, k).$$

If replication and segregation are independent then, from equations (A 1 and (A 2), the probability that there are j copies of plasmid 1 at birth given tha there were i copies at birth the generation before is

$$p_{i,j} = \begin{cases} \sum_{k=\max(i,j)}^{\min(N+i,N+j)} \dfrac{\binom{k-1}{i-1}\binom{2N-k-1}{N-i-1}\binom{k}{j}\binom{2N-k}{N-j}}{\binom{2N-1}{N-1}\binom{2N}{N}} & \text{for} \quad 1 \leqslant i \leqslant N-1 \\ 0 \quad \text{for} \quad i = 0 \quad \text{or} \quad i = N \quad \text{and} \quad i \neq j \\ 1 \quad \text{for} \quad i = 0 \quad \text{or} \quad i = N \quad \text{and} \quad i = j. \end{cases} \tag{A 3}$$

The matrix $(p_{i,j})$ was calculated by computer for N in the range 2–40. Thi allowed the calculation of the proportion of cells carrying both plasmids afte starting from various initial distributions of plasmids. We were also able to calcu late the steady state rate of segregation exactly. The limiting distribution of th number of plasmid 1 copies per cell at birth for the cells carrying both plasmids is uniform distribution. We show this by proving that

$$\sum_{i=1}^{N-1} p_{i,j} = \lambda \quad \text{for} \quad j = 1, 2, \ldots, N-1. \tag{A 4}$$

Thus λ is the ratio by which the proportion of cells carrying both plasmids de creases in each generation during steady state segregation. We calculate λ and hence, the steady state segregation rate. Substitution from equation (A 3) into th left hand side of equation (A 4) gives

$$\begin{aligned} \binom{2N-1}{N-1}\binom{2N}{N}\sum_{i=1}^{N-1} p_{i,j} &= \sum_{i=1}^{N-1}\sum_{k=\max(i,j)}^{\min(N+i,N+j)} \binom{k-1}{i-1}\binom{2N-k-1}{N-i-1}\binom{k}{j}\binom{2N-k}{N-j} \\ &= \sum_{k=j}^{N+j}\binom{k}{j}\binom{2N-k}{N-j}\sum_{i=1}^{k}\binom{k-1}{i-1}\binom{2N-k-1}{N-i-1} \end{aligned}$$

$$= \binom{2N-2}{N-2} \sum_{k=j}^{N+j} \binom{k}{j} \binom{2N-k}{N-j}$$

$$= \binom{2N-2}{N-2} S_j$$

the sum over i is the coefficient of

$$t^{N-2} \ (= t^{i-1+N-i-1}) \quad \text{in} \quad (1+t)^{2N-2} \ (= (1+t)^{k-1} \ (1+t)^{2N-k-1}).$$

us we need to show that S_j is independent of j and we do this by calculating S_j.
is the coefficient of $s^j t^{N-j}$ in $\mathscr{S}_j(s, t)$, where

$$\mathscr{S}_j(s, t) = \sum_{k=j}^{N+j} (1+s)^k \ (1+t)^{2N-k}$$

$$= (1+s)^j \ (1+t)^{N-j} \sum_{m=0}^{N} (1+s)^m \ (1+t)^{N-m}$$

$$= (1+s)^j \ (1+t)^{N-j} \ \frac{(1+s)^{N+1} - (1+t)^{N+1}}{s-t}$$

$$= (1+s)^j \ (1+t)^{N-j} \sum_{k=1}^{N+1} \binom{N+1}{k} \frac{s^k - t^k}{s-t}$$

$$= (1+s)^j \ (1+t)^{N-j} \sum_{k=1}^{N+1} \binom{N+1}{k} \sum_{m=0}^{k-1} s^m t^{k-m-1}.$$

us, $$S_j = \sum_{a=0}^{j} \sum_{b=0}^{N-j} \binom{j}{a} \binom{N-j}{b} \binom{N+1}{N+1-a-b}.$$

s is the coefficient of s^{N+1} in $(1+s)^{2N+1}$. Therefore

$$S_j = \binom{2N+1}{N+1}.$$

s is independent of j, so this proves that equation (A 4) is correct and the value
is

$$\lambda = \frac{(N-1)\,(2N+1)}{(N+1)\,(2N-1)}. \tag{A 5}$$

e half time for segregation ($t_{\frac{1}{2}}$) is given by

$$t_{\frac{1}{2}} = -\log_e 2 / \log_e \lambda. \tag{A 6}$$

pansion of $\log_e \lambda$ in powers of $1/N$ showed that $t_{\frac{1}{2}} \sim N \log_e 2$ as $N \to \infty$.

Random segregation

We also considered a random segregation model in which the $2N$ plasmid copies
dividing cell are distributed at random between the two daughter cells. The
bability of obtaining a daughter cell with s copies of plasmid 1 and t copies of
smid 2 if the parent has k copies of plasmid 1 and $2N-k$ copies of plasmid 2 at

division is

$$P(s,t|k,2N-k) = \left\{\binom{2N}{s+t}(\tfrac{1}{2})^{2N}\right\}\left\{\frac{\binom{k}{s}\binom{2N-k}{t}}{\binom{2N}{s+t}}\right\},$$

where the first term is the probability of producing a daughter cell with $s+$ plasmid copies and the second term is the probability of producing s plasmid copies and t plasmid 2 copies in the daughter cell at birth given that the daught cell receives a total of $s+t$ plasmid copies. This simplifies to

$$P(s,t|k,2N-k) = (\tfrac{1}{2})^{2N}\binom{k}{s}\binom{2N-k}{t} \quad \text{for} \quad \begin{cases}0 \leqslant s \leqslant k\\ 0 \leqslant t \leqslant 2N-k.\end{cases} \qquad \text{(A}$$

This segregation model produces plasmid-free cells at a rate of $(\frac{1}{2})^{2N}$ per gener tion. Taking these cells into account in the calculations is inconvenient so v restricted attention to cells that carried plasmids. This accounts for the normali ing term $2^{2N}-1$ that appears in the equations below. Combining equations (A and (A 7) shows that the probability that a plasmid-carrying cell at division co tains j copies of plasmid 1 given that the generation before there were i copies plasmid 1 at division is

$$q_{i,j} = \begin{cases} \dfrac{1}{2^{2N}-1}\displaystyle\sum_{s=1}^{\min(i,j)}\sum_{t=1}^{\min(2N-i,2N-j)}\frac{\binom{j-1}{s-1}\binom{i}{s}\binom{2N-j-1}{t-1}\binom{2N-i}{t}}{\binom{2N-1}{s+t-1}} \\ \qquad \text{for} \quad 1 \leqslant i \leqslant 2N-1, \quad 0 < j < 2N \\ 0 \quad \text{for} \quad i = 0, \quad j > 0 \quad \text{or} \quad i = 2N, \quad j < 2N \\ 1 \quad \text{for} \quad i = 0, \quad j = 0 \quad \text{or} \quad i = 2N, \quad j = 2N \\ \dfrac{2^{2N-i}-1}{2^{2N}-1} \quad \text{for} \quad 1 \leqslant i \leqslant 2N-1, \quad j = 0 \\ \dfrac{2^{i}-1}{2^{2N}-1} \quad \text{for} \quad 1 \leqslant i \leqslant 2N-1, \quad j = 2N. \end{cases} \qquad \text{(A}$$

We used a computer to calculate $q_{i,j}$ for N in the range 2–20 and used this find the segregation rates from a variety of starting distributions of the tw plasmids.

(iii) *Differential growth rates*

We set up differential equations for the number of cells carrying only plasmid (x), only plasmid 2 (y) and both plasmids (m). We assumed that cells carrying bo plasmids grew exponentially with rate constant k and segregated symmetrical into cells containing only one type of plasmid at a rate s. We assumed that ce carrying only plasmid 1 grew at a rate $k-e$ and that cells carrying only plasm 2 grew at a rate $k+d$. These assumptions gave the equations:

$$\begin{aligned}\dot{x} &= (k-e).x+s.m/2,\\ \dot{y} &= (k+d).y+s.m/2,\\ \dot{m} &= k.m-s.m.\end{aligned} \qquad \text{(A}$$

The set of equations (A 9) is linear and easily solved analytically. We inserted measured parameters of the initial values of x, y and m into the equations. We measured values of k and used three choices of the values of d and e to obtain desired value for s.

a) No correction: $d = e = 0$.

b) Cells carrying both plasmids grew at the same rate as cells carrying plasmid alone: $e = 0$ and the value of d (> 0) was chosen to give the observed 5% ference in growth rates.

c) Cells carrying both plasmids grew at the same rate as cells carrying plasmid alone: $d = 0$ and the value of e (> 0) was chosen to give the observed 5% ference in growth rates.

Democratic replication

We considered a 'democratic' replication model in which each plasmid copy eplicated once per generation. We used the equal number segregation model uation (A 2)). This gave the probability that there were j copies of plasmid 1 in ell at birth given that there were i copies of plasmid 1 at birth one generation ore as

$$_{j} = \begin{cases} \dfrac{\binom{2i}{j}\binom{2N-2i}{N-j}}{\binom{2N}{N}} \\ \quad \text{for} \quad 1 \leqslant i \leqslant N-1 \quad \text{and} \quad \max(0,\, 2i-N) \leqslant j \leqslant \min(2i,\, N) \quad \text{(A 10)} \\ 1 \quad \text{for} \quad i = 0, \quad j = 0 \quad \text{or} \quad i = N, \quad j = N \\ 0 \quad \text{otherwise.} \end{cases}$$

We used computer programs to calculate $(r_{i,\,j})$ for N in the range 2–20. We ulated the steady state segregation rate by computer modelling of segregation ng the transition probabilities $(r_{i,\,j})$ and also modelled the situation where cells t with 1 copy of plasmid 1 and $N-1$ copies of plasmid 2.

Random pool replication with unequal chances of replication

To obtain equation (A 1) we assumed that the probability of replication was the e for every plasmid copy in a cell. Here we consider a model in which replica- is biased in favour of one plasmid so that the probability of replicating plasmid α times greater than that of replicating plasmid 2. Thus if there were s ies of plasmid 1 in a cell and t copies of plasmid 2, the probability that plasmid ould be replicated next is $\alpha s/(\alpha s+t)$ and the probability that plasmid 2 is icated next is $t/(\alpha s+t)$. We could not obtain an equation analogous to equation 1) because each order of replicating the two plasmids has a different probability. were, however, able to calculate the transition probabilities by using a com- er program that summed over all the possible orders of replication. We used to study the effect of different values of α on the degree of bias in segregation r starting from an equal number of copies of the two plasmids.

We wish to thank the Medical Research Council for a Training Grant (to J.C.) and Project Grant (to P.B.).

REFERENCES

ABE, M. (1974). The replication of prophage P1 DNA. *Molecular and general Genetics* **132**, 63–72.

ACHTMAN, M., WILLETTS, N. & CLARK, A. J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterisation of transfer-deficient mutants. *Journal of Bacteriology* **106**, 529–538.

ALFARO, G. & WILLETTS, N. (1972). The relationship between the transfer systems of some bacterial plasmids. *Genetical Research* **20**, 279–289.

ANDRESDOTTIR, V. & MASTERS, M. (1978). Evidence that F′*lac* replicates asynchronously during the cell cycle of *Escherichia coli* B/r. *Molecular and general Genetics* **163**, 205–212.

BARTH, P. T., DATTA, N., HEDGES, R. W. & GRINTER, N. J. (1976). Transposition of deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistances from R483 to other replicons. *Journal of Bacteriology* **125**, 800–810.

BAZARAL, M. & HELINSKI, D. R. (1970). Replication of a bacterial plasmid and an episome in *Escherichia coli*. *Biochemistry* **9**, 399–406.

CABELLO, F., TIMMIS, K. & COHEN, S. N. (1976). Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. *Nature* **259**, 285–290.

COLLINS, J. & PRITCHARD, R. H. (1973). Relationship between chromosome replication and F′*lac* episome replication in *Escherichia coli*. *Journal of Molecular Biology* **78**, 143–155.

COOPER, S. & HELMSTETTER, C. E. (1968). Chromosome replication and the division cycle of *Escherichia coli* B/r. *Journal of Molecular Biology* **31**, 519–540.

CULLUM, J., COLLINS, J. F. & BRODA, P. (1978). Factors affecting the kinetics of progeny formation with F′*lac* in *Escherichia coli* K12. *Plasmid* **1**, 536–544.

DATTA, N. & BARTH, P. T. (1976). Compatibility properties of R483, a member of the plasmid complex. *Journal of Bacteriology* **125**, 796–799.

DE VRIES, J. & MAAS, W. K. (1973). Description of an incompatibility mutant of *Escherichia coli*. *Journal of Bacteriology* **115**, 213–220.

DE VRIES, J. K., PFISTER, A., HAENNI, C., PALCHAUDHURI, S. & MAAS, W. (1975). F incompatibility. In *Microbiology 1974* (ed. D. Schlessinger), pp. 166–170. Washington: ASM.

DONACHIE, W. D., BEGG, K. J. & VICENTE, M. (1976). Cell length, cell growth and cell division. *Nature* **264**, 328–333.

DOWMAN, J. E. (1973). Implications of stochastic inheritance. *Journal of theoretical Biology* **39**, 55–72.

DURKACZ, B. W. & SHERRATT, D. J. (1973). Segregation kinetics of colicinogenic factor ColE1 from a bacterial population temperature-sensitive for DNA polymerase 1. *Molecular and general Genetics* **121**, 71–75.

ECHOLS, H. (1963). Properties of F′ strains of *Escherichia coli* superinfected with F-lactose and F-galactose episomes. *Journal of Bacteriology* **85**, 262–268.

ENGBERG, B., HJALMARSSON, K. & NORDSTRÖM, K. (1975). Inhibition of cell division of *Escherichia coli* K12 by the R-factor R1 and copy mutants of R1. *Journal of Bacteriology* **124**, 633–640.

FINKELSTEIN, M. & HELMSTETTER, C. E. (1977). Cell cycle analysis of F′*lac* replication in *Escherichia coli* B/r. *Journal of Bacteriology* **132**, 884–895.

GUSTAFSSON, P. & NORDSTRÖM, K. (1975). Random replication of the stringent plasmid R1 in *Escherichia coli* K12. *Journal of Bacteriology* **123**, 443–448.

GUSTAFSSON, P., NORDSTRÖM, K. & PERRAM, J. W. (1978). Selection and timing of replication of plasmids R1*drd-19* and F′*lac* in *Escherichia coli*. *Plasmid* **1**, 187–203.

HASHIMOTO-GOTOH, T. & SEKIGUCHI, M. (1977). Mutations to temperature-sensitivity in plasmid pSC101. *Journal of Bacteriology* **131**, 405–412.

ISHII, K., HASHIMOTO-GOTOH, T. & MATSUBARA, K. (1978). Random replication and random assortment model for plasmid incompatibility. *Plasmid* **1**, 435–446.

JACOB, F., BRENNER, S. & CUZIN, F. (1963). On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symposia on Quantitative Biology* **28**, 329–347.

MIESON, A. F. & BERGQUIST, P. L. (1977). Role of *dnaB43* in F′-plasmid incompatibility. *Molecular and general Genetics* **150**, 161–170.

LINE, B. C. (1974). Mechanism and biosynthetic requirements for F plasmid replication in *Escherichia coli*. *Biochemistry* **13**, 139–146.

ACFARREN, A. C. & CLOWES, R. C. (1967). A comparative study of two F-like colicin factors, ColV2 and ColV3, in *Escherichia coli* K12. *Journal of Bacteriology* **94**, 365–377.

AY, J. W., HOUGHTON, R. H. & PERRET, C. J. (1964). The effect of growth at elevated temperatures on some heritable properties of *Staphylococcus aureus*. *Journal of general Microbiology* **37**, 157–169.

VICK, R. P. & BRODSKY, R. (1972). Studies on plasmid replication. I. Plasmid incompatibility and establishment in *Staphylococcus aureus*. *Journal of Molecular Biology* **68**, 285–302.

VICK, R. P. & HOPPENSTEADT, F. C. (1978). On plasmid incompatibility. *Plasmid* **1**, 421–434.

VICK, R. P. & SCHWESINGER, M. (1976). Independence of plasmid incompatibility and replication control functions in *Staphylococcus aureus*. *Nature* **262**, 623–626.

VICK, R., WYMAN, L., BOUANCHAUD, D. & MURPHY, E. (1975). Plasmid life cycles in *Staphylococcus aureus*. In *Microbiology 1974* (ed. D. Schlessinger), pp. 115–129. Washington: ASM.

ITCHARD, R. H., BARTH, P. T. & COLLINS, J. (1969). Control of DNA synthesis in bacteria. *Symposia of the Society for general Microbiology* **19**, 263–297.

WND, R. (1969). Replication of a bacterial episome under relaxed control. *Journal of Molecular Biology* **44**, 387–402.

N BLAS, F., THOMPSON, R. & BRODA, P. (1974). An *Escherichia coli* K12 mutant apparently carrying two autonomous F-prime factors. *Molecular and general Genetics* **130**, 153–163.

MMIS, K. N., ANDRES, I. & SLOCOMBE, P. M. (1978). Plasmid incompatibility: cloning analysis of an *incFII* determinant of R6–5. *Nature* **273**, 27–32.

LIN, B. E. & NORDSTRÖM, K. (1975). Plasmid incompatibility and control of replication: copy mutants of the R-factor R1 in *Escherichia coli* K12. *Journal of Bacteriology* **124**, 641–649.

ITTLE, P. (1970). *Probability*. Penguin Books Ltd.

JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 741-746

Vol. 117, No. 2
Printed in U.S.A.

Modified Map Positions for *lac* and the *pro* Markers in *Escherichia coli* K-12

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Received for publication 23 November 1973

Interrupted mating experiments were performed with Hfr strains H and C and three *leu lac purE* recipient strains derived from a common parent and carrying, respectively, the *proA*$^-$, *proB*$^-$, and *proC*$^-$ mutations. It was concluded that if *leu* is placed at 1.5 min and *purE* at 12 min from *thr*, the origin on the Taylor-Trotter map, *lac* is at about 7.5 min and the *pro* genes are at about 6.0, 6.6, and 8.4 min, respectively. Both conjugational and transductional data suggest that the strain carrying the *proB*$^-$ mutation also carries a second mutation close to the *proA* site which independently confers a Pro$^-$ phenotype. The times before the onset of transfer of chromosomal deoxyribonucleic acid by both Hfr strains B4 and B8 were approximately 3 min

When Hfr and F$^-$ cultures of *Escherichia coli* K-12 are mixed, there is an interval (3 to 5 min), the "origin entry time" (14), before the first donor markers are transferred to the recipient cells. In connection with experiments to be described in the succeeding paper (4), it was necessary to establish this interval for two further Hfr strains, B4 and B8.

There are three *pro* genes close to *lac* (6, 20; L. . Charamella and R. Curtiss III, Bacteriol. Proc., p. 27, 1966) (see Fig. 1). The origins of Hfr strains B4 and B8 lie between *proB* and *lac* and *proC* and *lac*, respectively (see Results). Their approximate origin entry times were obtained by comparing the times of entry (t_E) of the markers they transferred early and map distances in this region. The interval between two markers, as determined in mating experiments, varies, in part depending upon the strains and conditions used (4). The object in these experiments was, therefore, to determine relative intervals between markers within the *leu-purE* region, with reference to these outside markers. These distances were determined in interrupted matings between Hfr strains H and C and three *leu lac purE* recipient strains of common ancestry carrying, respectively, *proA*$^-$, *proB*$^-$, and *proC*$^-$ mutations. It became clear that the published map positions required revision. Moreover, the "*proB*$^-$" strain appeared to carry two mutations, each of which gave a Pro$^-$ phenotype. These observations may clarify some of the conflicting results obtained earlier for the *proB* locus (20).

MATERIALS AND METHODS

Strains. The origins of transfer of the Hfr strains used are shown in Fig. 1; other particulars concerning strains are presented in Table 1.

Mating conditions. All growth and matings were conducted in L broth at 37 C. Initial cultures were grown up and kept on ice; subcultures were diluted into fresh broth and grown for two generations before mating so that each of a pair of cultures reached 40 U, as measured with a Klett-Summerson photometer (about 2×10^8 cells/ml) simultaneously. Samples of 10 ml of donor and recipient strains were then mixed and incubated in a 100-ml conical flask in a New Brunswick rotary shaking water bath (100 rpm). Matings were done in sets to standardize mating conditions (for instance temperature and shaking) and the state of the cultures. Samples, usually 0.1 ml, were withdrawn without interrupting shaking, and diluted (usually $\times 100$) into M9 buffer; and a 0.1-ml sample was mixed with 3 ml of molten soft-water agar containing 0.1 ml of broth. Mating was interrupted by 15-s agitation by the method of Low and Wood (16). The beginning of the agitation defined the time of interruption. The contents were poured onto selective minimal agar plates, which were then incubated for 2 days.

Scoring for entry of *lac*$^+$ was generally done by inoculating clones of another progeny class on the same selective medium, letting them grow overnight, and replica plating for the co-inheritance of *lac*$^+$.

The discontinuous nature of the data (15, 19, 21) makes the definition of precise entry curves impossible. The t_E values used here were obtained by extrapolating back from the first two points considered to differ significantly from zero.

Transduction. Transductional crosses with phage P1*kc* were performed by the procedures of Yanofsky and Lennox (23).

FIG. 1. *The thr-purE region with the origins of Hfrs H, B4, B8, and C. The upper row of numbers refers to the positions assigned by Taylor and Trotter (20) to the different markers. The lower row gives those proposed in this communication.*

TABLE 1. *Strains employed*[a]

Strain	Genotype	Source or reference
B4	*metB* λ^- λ^R *str*$^+$	3
B8	*metB* λ^- λ^R *str*$^+$	3
ED935 (= Hfr H)	λ^- *str*$^+$	Edinburgh collection
ED947 (= Hfr C)	*metB str*$^+$	Edinburgh collection
χ462	*proA leu metE lys trp purE lac str*	2
χ474	*proB leu metE lys trp purE lac str*	2
χ478	*proC leu metE lys trp purE lac str*	2
AB1157	*thr leu argE his proA str*	1, 12

[a] The nomenclature is that recommended by Taylor and Trotter (20).

RESULTS

To verify the position of *leu* with respect to *thr*, the mutation that defines 0 min on the *E. coli* map (20), the numbers of Thr$^+$ clones were determined among the Leu$^+$ progeny in successive samples selected in interrupted matings between Hfr strains C and B4 and strain AB1157. In both crosses, the displacement between the entry curves for *leu*$^+$ and *thr*$^+$ was about 1 min. Since Taylor and Trotter (20) have recently assigned *leu* to 1.5 min, on the basis of various transductional data, we have based the calculations made here on this figure. Similarly, we have used their value of 12 min for the position of the other reference marker, *purE*.

Hfr strains H and C were used in crosses with strains χ462, χ474, and χ478 (three strains, generously provided by R. Curtiss III, that were derived from a common *leu purE lac* strain, χ454) to determine the t_E values for *leu*$^+$, *pro*$^+$, and *purE*$^+$. The t_E values for *lac*$^+$ were determined by scoring the appropriate classes of Pro$^+$ and PurE$^+$ progeny obtained. This was done so that the entry curves for two markers (i.e., *lac*$^+$ and *pro*$^+$ or *purE*$^+$) could be constructed using the same samples. Data from four crosses are shown in Fig. 2, and the averaged t_E values are given in Table 2.

In all of these six types of cross, the transfer times for the whole *leu-purE* region are very similar, with an average value of 11.2 min. This compares with the map distance of 10.5 min given by Taylor and Trotter (20). For the purpose of discussion, the t_E values for *lac* and *pro*, as determined in each type of cross, are normalized to give positions on the basis of the total distance between the reference markers *leu* and *purE* being 10.5 min. The distance from *leu* to *proC* was 6.6 and 7.2 min in the Hfr H and HfrC crosses, respectively; these figures place *proC* at approximately 1.5 + 6.9 = 8.4 min.

Crosses between strains Hfr H and χ462 and χ474 (Table 2) supported the conclusion (6, 20) that *proA* and *proB* are at sites some distance apart (cf. reference 5). We can assign *proA* to 1.5 + 4.2 = 5.7 min and *proB* to 1.5 + 5.1 = 6.6 min. A more critical demonstration of their separate locations comes from the construction of *lac*$^+$ entry curves by the analysis of the Pro$^+$ progeny in these two crosses. In three crosses with strain χ462, the displacements between the *proA*$^+$ and *lac*$^+$ curves were 2.3, 2.0, and 1.7 min, and in two crosses with strain χ474 the corresponding displacements were 0.5 and 0.7 min.

The average of all the t_E determinations for *lac*$^+$ suggests that its proper place is at 1.5 + 6.0 = 7.5 min (standard error = 0.4 min). We conclude that the published marker assign-

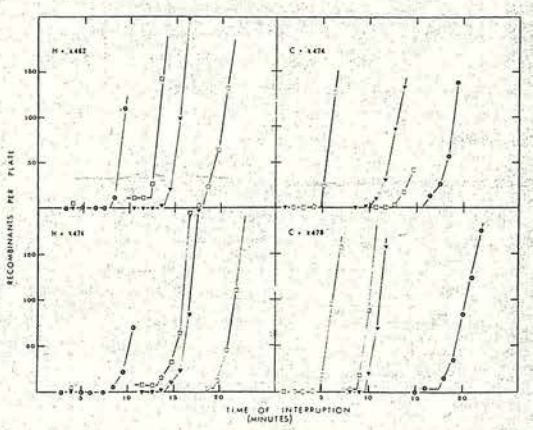

FIG. 2. *Recombinant formation in interrupted mating experiments. Symbols:* ●, *Leu*$^+$ *progeny;* □, *Pro*$^+$ *progeny;* ○, *Pur*$^+$ *progeny;* ▼, *Lac*$^+$ *progeny or (in the strain Hfr C* × *strain χ474 cross), Pur*$^+$*Lac*$^+$ *progeny. In the strain Hfr C* × *χ474 cross, the Pro*$^+$ *curve was constructed by patching the Pur*$^+$*Lac*$^+$ *progeny and determining the number of Pro*$^+$ *clones. In the other crosses, the Pro*$^+$ *progeny were patched and replica-plated to determine the number of Lac*$^+$ *clones.*

ments for *lac* and the *pro* markers relative to the *leu* and *purE* markers are significantly in error. The results of the strain Hfr C × strain χ462 crosses place *proA* at 6.4 min. Combining the data from the strain Hfr H and Hfr C crosses with strain χ462, we can therefore place *proA* at approximately 6.0 min. However, the strain Hfr C × χ474 crosses place *proB* at 6.1 min; that is, in contrast to the conclusion from the crosses between strains Hfr H and χ474, *proB* appears to lie close to *proA*. Moreover, when an entry curve was constructed for *pro*$^+$ by testing directly selected Lac$^+$ progeny from the latter cross, the displacement between the *lac*$^+$ and *pro*$^+$ curves was 1.9 min, compared with an average of 0.6 min when entry of *pro*$^+$ and *lac*$^+$ were compared in the strain Hfr H × strain χ474 cross. The simplest explanation would be that the Pro$^-$ phenotype of strain χ474 is due to a double mutation, one at the *proB* site and the other in a gene that lies close to *proA* or even in *proA* itself. In interrupted matings Pro$^+$ progeny would then only start to appear when both *proA* and *proB* had been transferred.

Support for this view came from transduction experiments using phage P1*kc*. In the first experiment, lysates from Hfr strains H and C and from strain W1655, the parent of strain Hfr B4, were used to transduce strains χ462, χ474, and χ478 (Table 3); Leu$^+$, Pro$^+$ and Pro$^+$Lac$^+$ progeny were selected. The results with the three donor strains are similar; in each case, the yields of Pro$^+$ progeny relative to Leu$^+$ progeny were approximately similar when strains χ462 (*proA*) and χ478 (*proC*) were the recipients, but much greater than the yield of Pro$^+$ progeny obtained with strain χ474 (*proB*). Furthermore, only strain χ478 gave Pro$^+$Lac$^+$ progeny. These observations suggest that *proA*$^-$ and *proC*$^-$ used here are single mutations, where *proC* but not *proA* is co-transducible with *lac*, and that the "*pro*$^-$" in our culture of strain χ474 is a double mutation in which the two mutations can be transduced, but they cannot together be co-transduced with *lac*. We can calculate by the method of Wu (22) that *proC* and *lac* are about 0.8 min apart. The ratio of Pro$^+$ progeny yields 0.13/0.49 with strains χ474 and χ462 as recipients is that expected on Wu's formula if the two postulated mutations in strain χ474 were 0.7 min apart. This is reasonably consistent with the hypothesis that the two mutations occur at the *proA* and *proB* loci.

In the second experiment (data not shown), strains χ462 and χ474 were transduced with lysates made by two cycles of growth on Leu$^+$

TABLE 2. *Average t_E values from crosses using Hfr strains H and C, and the F$^-$ strains χ462, χ474 and χ478*[a]

Cross	No. of expt	Avg t_E values						Avg measured interval (*leu*$^+$–*purE*$^+$)	Calculated interval *leu–pro*[b]			Avg measured displacement (*pro*$^+$–*lac*$^+$)	Calculated interval (*leu–lac*)[b]
		leu$^+$	*proA*$^+$	*proB*$^+$	*lac*$^+$	*proC*$^+$	*purE*$^+$		*proA*	*proB*	*proC*		
HfrH × χ462	3	8.3	12.5		14.5		18.8	10.5	4.2			2.0	6.2
HfrH × χ474	2	8.4		14.0	14.6		19.7	11.3 } 11.0		5.2 } 5.1		0.6	5.8
	1	8.5		13.5			19.0	10.5 }		5.0 }			
HfrH × χ478	3	8.8				15.6	19.7	10.9			6.6		
HfrC × χ462	1	14.8	11.2		9.7		4.8	10.0 } 10.6	3.8 } 4.9			1.5	5.4
	3	15.6	10.1				4.8	10.8 }	5.3 }				
HfrC × χ474	2	16.6		12.2	10.3		4.9	11.7 } 11.5		3.9 } 4.6		1.9	5.7
	3	16.2		10.7			4.8	11.4 }		5.1 }			
HfrC × χ478	4	17.2			9.7	8.6	4.7	12.5			7.2	1.1	6.3

[a] The t_E values of *lac*$^+$ were determined by replication of patched Pro$^+$ or PurE$^+$ progeny, except that in one of the Hfr H × χ474 crosses Lac$^+$ progeny were selected directly; these were then used to determine the t_E for *pro*$^+$.

[b] Calculated on the basis of the length of the *leu–purE* segment being 10.5 min (20).

TABLE 3. *Relative yields of Pro⁺ and Pro⁺Lac⁺ progeny, normalized against the yields of Leu⁺ progeny, in pairwise transductional crosses*

Recipient strain	Progeny class	Donor strain			Avg
		HfrH	HfrC	W1655	
χ462	Pro⁺	0.50	0.54	0.42	0.49
	Pro⁺Lac⁺	<0.001	<0.001	<0.001	<0.001
χ474	Pro⁺	0.12	0.15	0.11	0.13
	Pro⁺Lac⁺	<0.0005	<0.0005	<0.0005	<0.0005
χ478	Pro⁺	0.41	0.51	0.37	0.43
	Pro⁺Lac⁺	0.08	0.08	0.10	0.09
AB1157	Pro⁺	0.46	0.50	0.46	0.47

derivatives of strains χ462 and χ474 obtained in the above experiment. In the crosses with strain χ462 derivative as the donor and strain χ474 as the recipient, and the strain χ474 derivative as the donor and strain χ462 as the recipient, few if any Pro⁺ progeny were obtained (both strains give rise spontaneously to Pro⁺ clones at a frequency of 10^{-7} to 10^{-8} [11, 13]). This supports the view that strain χ474 carries a mutation at or very close to *proA*.

Matings between strains B4 and χ462 gave average t_E values of 4.0 and 9.7 min for *proA*⁺ and *leu*⁺, respectively (Table 4). The distance between these markers (5.7 min) compares with values of 4.2 and 4.9 min with Hfr strains H and C. Since we cannot measure the transfer time for the *leu-purE* region with Hfr strain B4, we can only conclude that in this strain *proA* bears approximately the same relation to *leu* as in Hfr strains H and C. Among the Leu⁺ progeny of this cross, 39% were Pro⁺ and none out of 100 Pro⁺ clones was Lac⁺. We conclude that Hfr strain B4 does not transfer *lac*⁺ early.

In the analagous cross between strains Hfr B4 and χ474, the yield of Pro⁺ progeny was much lower. This is illustrated by the fact that only 4 among 320 Leu⁺ progeny were also Pro⁺; this would be expected for a marker transferred very close to the leading end of the Hfr chromosome (9, 14). The earlier conclusion that Hfr strain B4 does not transfer *lac*⁺ early was strengthened by the observation that among 100 Pro⁺ progeny none was Lac⁺. The fact that the average t_E value for *pro*⁺ here (Table 4) is greater than that for *proA*⁺ (from the previous experiment) is probably due to the relatively low yield of Pro⁺ progeny obtained in the cross between strains Hfr B4 and χ474.

If *lac* in Hfr strain B4 is in the same position relative to *proA* and *proB* as in Hfr strains H and C (that is, at 7.5 min on a normalized map), the origin of Hfr strain B4 lies between 6.6 and 7.5 min. If *proA* is at 6.0 min, the t_E value for *proA* with Hfr strain B4 yields an origin entry time of 4.0 − (0.6 to 1.5) = 2.5 to 3.4 min.

Matings between strains Hfr B8 and χ478 gave average t_E values for *proC*⁺ and purE⁺ o 3.9 and 8.2 min, respectively. This implies a distance of 4.3 min between *proC* and *purE* compared with distances using Hfr strains H and C of 3.9 and 3.3 min, respectively. W conclude that in Hfr strain B8 *proC*⁺ bear approximately the same relation to *purE* as i Hfr strains H and C. Only 6% of the Pur progeny tested were Pro⁺, and out of 100 Pro progeny none was Lac⁺. We conclude that th origin of Hfr strain B8 is between *lac* and *proC* that is, between 7.5 and 8.4 min on a normalize map. If the t_E value for *proC* is 3.9 min, we ca conclude that the origin entry time is betwee 3.9 min and 3.9 − (8.4 − 7.5) = 3.0 min. Usin the t_E value for *purE* of 8.2 min, we obtain value of between 8.2 − (12 − 8.4) = 4.6 min an 8.2 − (12 − 7.5) = 3.7 min.

DISCUSSION

By taking frequent samples and performin several sets of crosses under carefully standard ized conditions, it is possible to obtain qui reproducible t_E values and to map markers within about 0.5 min. Also, by using the numb of progeny of an unselected class among th selected progeny to construct a separate ent curve, one can measure accurately the distan between two markers by using a single cros These data can be most usefully employed map genes relative to outside reference marke rather than to attempt absolute determinatio in minutes of transfer time, since the latter a likely to vary more with the precise strains a methods used. It is clear (Table 2) that there some variation within the data from sets crosses. Nevertheless, the consistency betwe the determinations for *lac* gives confidence th *lac* can now be placed rather accurately at min if the positions for *leu* and *purE* are tak as 1.5 and 12 min (20). This approach

TABLE 4. *Average t_E values obtained in crosses w Hfr strains B4, B8, and C*

Cross	t_E		
	leu⁺	*pro*⁺	*purE*
Hfr B4 × χ462	9.7	4.0	
Hfr B4 × χ474	9.3	5.0	
Hfr B4 × AB1157	10.7	4.8	
Hfr C × AB1157	16.6	11.9	
Hfr B8 × χ478		3.9	8.2

relevant to the general problem of mapping intervals at and above the limits amenable to phage P1 transduction.

These experiments revealed an anomaly with respect to strain χ474 since crosses between this strain and Hfr strains H and C placed *proB*$^+$ in these Hfr strains about 1 min from *proA* and very close to it, respectively. The suggestion that this marker lies in different positions depending upon the Hfr strain used is supported by the intervals between the t_E curves for "*proB*" and *lac* in these crosses. One possibility is that our culture of strain χ474 is a double mutant carrying *proB* and another mutation that lies close to *proA*, and that to become Pro$^+$ it must receive the + alleles for both markers. The basis of this double mutation could either be two-point mutations or a deletion extending near to *proA*, if not into or beyond it.

The transductional data are consistent with this hypothesis. In the first place, the yield of ProB$^+$ transductants (Table 3) was unexpectedly low for a single mutation, but about that expected for two markers about 0.7 min apart. Second, no progeny were obtained in transductional crosses between "proA$^+$ proB$^-$" and proA$^-$ proB$^-$ strains, a result that would not have been expected if each strain had been singly mutant and the mutations were some distance apart (5). It suggests again that the second mutation in strain χ474 would have to be close to (or even at) the *proA* locus.

The proposal that our culture of strain χ474 has become a double mutant would also resolve an apparent paradox. On the one hand, we know from our results with matings between Hfr strain B4 and strain χ474 that (as shown by Curtiss [6]) an allele transferred very close to *lac* (i.e., the *proB* locus) is necessary for Pro$^+$ progeny to arise. On the other hand, unlike Curtiss and Charamella (Bacteriol. Proc., p. 27, 1966) and Roberts and Reeve (17), we were unable to observe cotransduction to give Pro$^+$Lac$^+$ progeny; this could be explained if a third locus, in the *proA* region, now has to be introduced together with *proB*$^+$ and *lac*$^+$.

An alternative explanation for the observations with strain χ474 can be considered, namely that *proB*$^+$ has become closer to *proA*$^+$ in Hfr C. This possibility is unlikely, since the yield of Pro$^+$ progeny of strain χ474 is lowered to an equal extent in transductions using lysates from strains Hfr C, Hfr H and W1655, and because co-transduction between *proB* and *lac* was not observed in any of these crosses.

It therefore appears that since its initial isolation and characterization as a *proB* mutant (6) strain χ474 has become altered. Since Condamine (5) has on the basis of complementation tests concluded that another initially *proB* strain, χ278 (6), lacked activity for both the *proA* and *proB* cistrons, this raises the question of whether *proB* strains in general acquire a selective advantage in undergoing the postulated secondary mutation. It could for instance be that *proB*$^-$ strains accumulate a toxic intermediate.

The double mutation model, if correct, has two obvious general implications. The first is that in conjugational mapping it is important to map a marker in both directions. The second is that it cannot be assumed that mutant strains remain genotypically unchanged during routine stock transfers. This point has been made in a general context by Curtiss and his colleagues (7, 8) among others.

A value of 3 min seems a reasonable estimate for the time when chromosomal, as distinct from F, deoxyribonucleic acid starts to enter the recipient cell from Hfr strains B4 and B8. Since the F particle has a molecular weight of about 62×10^6 (18) compared with a molecular weight of the *E. coli* chromosome of about 2.7×10^9 (12), we can calculate that it should take 2 min of actual transfer time to be transferred. Only when we know the amount of F that is actually transferred early (which may vary in different Hfr strains [18]) shall we be able to make a correction to obtain true entry times for Hfr strains.

ACKNOWLEDGMENT

I thank Rosemary Pringle for her excellent assistance.

LITERATURE CITED

1. Bachmann, B. J. 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. Berg, C. M., and R. Curtiss III. 1967. Transposition derivatives of an Hfr strain of *Escherichia coli* K12. Genetics **56**:503–525.
3. Broda, P. 1967. The formation of Hfr strains in *Escherichia coli* K12. Genet. Res. Camb. **9**:35–47.
4. Broda, P., and J. F. Collins. 1974. Gross map distances and Hfr transfer times in *Escherichia coli* K-12. J. Bacteriol. **117**:747–752.
5. Condamine, H. 1971. Mutants des voies de biosynthèse et de degradation de la proline chez *E. coli* K12. Ann. Inst. Pasteur **120**:9–22.
6. Curtiss, R., III. 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
7. Curtiss, R., III. 1969. Bacterial conjugation. Annu. Rev. Microbiol. **23**:69–136.
8. Curtiss, R., III, L. J. Charamella, D. R. Stallions, and J. A. Mays. 1968. Parental functions during conjugation in *Escherichia coli* K-12. Bacteriol. Rev. **32**:320–348.
9. Glansdorff, N. 1967. Pseudoinversions in the chromosome of *Escherichia coli* K-12. Genetics **55**:167–179.
10. Howard-Flanders, P., E. Simson, and L. Theriot. 1964. A

locus that controls filament formation and sensitivity to radiation in *Escherichia coli* K-12. Genetics **49**:237–246.
11. Itikawa, H., S. Baumberg, and H. J. Vogel. 1968. Enzymic basis for a genetic suppression: accumulation and deacylation of N-acetyl γ-semialdehyde in enterobacterial mutants. Biochim. Biophys. Acta, **159**:547–550.
12. Klotz, L. C., and B. H. Zimm. 1972. Size of DNA determined by viscoelastic measurements. Results on bacteriophages, *Bacillus subtilis* and *Escherichia coli*. J. Mol. Biol. **72**:779–800.
13. Kuo, T-T., and B. A. D. Stocker. 1969. Suppression of proline requirement of *proA* and *proAB* deletion mutants in *Salmonella typhimurium* by mutation to arginine requirement. J. Bacteriol. **98**:593–598.
14. Low, B. 1965. Low Recombination frequency for markers very near the origin in conjugation in *E. coli*. Genet. Res. Camb. **6**:496–473.
15. Low, B. 1973. Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. J. Bacteriol. **113**:798–812.
16. Low, B., and T. H. Wood. 1965. A quick and efficient method of interruption of bacterial conjugation. Genet. Res. Camb. **6**:300–303.
17. Roberts, L. M., and E. C. R. Reeve. 1970. Two mutations giving low level streptomycin resistance in *Escherichia coli* K-12. Genet. Res. **16**:359–365.
18. Sharp, P. A., M. Hsu., E. Ohtsubo., and N. Davidson. 1972. Electron microscope heteroduplex studies of sequence relations among plasmids of *E. coli*. I. Structure of F prime factors. J. Mol. Biol. **71**:471–497.
19. Taylor, A. L., and M. S. Thoman. 1964. The genetic map of *Escherichia coli* K12. Genetics **50**:659–677.
20. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* strain K-12. Bacteriol. Rev. **36**:504–524.
21. Wood, T. H. 1968. Effects of temperature, agitation, and donor strain on chromosome transfer in *Escherichia coli* K-12. J. Bacteriol. **96**:2077–2084.
22. Wu, T. T. 1966. A model for three point analysis of random general transduction. Genetics **54**:405–410.
23. Yanofsky, C., and E. S. Lennox. 1959. Transduction and recombination study of linkage relationships among the genes controlling tryptophan synthesis in *Escherichia coli*. Virology **8**:425–447.

JOURNAL OF BACTERIOLOGY, Feb. 1974, p. 747-752
Vol. 117, No. 2

Gross Map Distances and Hfr Transfer Times in *Escherichia coli* K-12

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Received for publication 28 August 1973

Hfr strains B4 and B8 transfer the *Escherichia coli* chromosome in opposite directions, each transferring *lac*$^+$ as the last known marker. They were mated in concurrent crosses with the *proA leu metE lys trp purE lac* strain χ462. Analysis of the time of entry values for these markers showed that Hfr strain B8 transfers the whole chromosome more rapidly than does Hfr strain B4. In both crosses, the rate of transfer observed decelerates. If deceleration occurs as a function of the amount of chromosome transferred, the data are consistent with the markers examined being very accurately placed on the Taylor-Trotter map of the *E. coli* K-12 genome.

The basis of the well-filled linkage map of the *Escherichia coli* K-12 chromosome (10) was the location of a number of markers by interrupted mating experiments (9). Other markers have been fitted into this map by using either conjugation or transduction as the tool. There is little doubt that this ordering of markers is generally correct, and this map has proved of the greatest value to all workers using *E. coli*. However, there are a priori grounds for questioning the overall relative dimensions, since positioning the original "landmarks" involved using several assumptions that had not, at that time or indeed subsequently, been tested adequately.

These assumptions include the following: (i) that different Hfr strains and, in particular, sibling Hfr strains transfer the chromosome at similar rates; (ii) that a given Hfr strain transfers its chromosome so that the intervals between the times of entry (t_E) of successive markers are proportional to the distances between them; (iii) that different recipient strains can accept chromosomal deoxyribonucleic acid (DNA) at the same rate; (iv) that the mating conditions and methods of deriving entry times from the data thus obtained in different laboratories yield comparable entry times. Wood (11), studying the physical conditions affecting mating parameters, concluded that in the *thr-his* region (38.5 min on the current map) chromosome transfer did not vary in rate by more than 10%.

The sibling Hfr strains B4 and B8 transfer the chromosome in opposite directions (see Fig. 1), and each transfers *lac*$^+$ as the last known marker (2, 3). In the main experiments to be described, the t_E values for a number of different markers, including *lac*$^+$, were determined by using a single recipient strain in concurrent matings with these Hfr strains. By relating the t_E values obtained in these crosses to the published map, it was possible to test whether the dimensions of this linkage map are compatible with the kinetics of chromosome transfer.

MATERIALS AND METHODS

Particulars of the strains used are given in Table 1. Hfr strains B4 and B8 have origins of transfer that are represented in Fig. 1.

Matings were performed by the procedures described in the preceding paper (3), with the following additional detail. About 15 min after beginning the matings, a 1-ml sample was diluted into 24 ml of prewarmed broth, using a 10-ml pipette to minimize shear. This diluted culture was used for determining t_E values of later markers; samples of either 0.1 or 0.2 ml were withdrawn and pipetted directly into soft agar for interruption.

Except for earliest markers, selection was always for more than one marker. For instance, selection for the transfer of the late marker *purE*$^+$ by strain Hfr B4 was accompanied by simultaneous selection for transfer of *leu*$^+$ (an early marker) and *lys*$^+$ (a middle marker). The purpose of the procedure was (i) to minimize the growth of revertants in the recipient population, and (ii) to ensure that the marker had indeed been transferred through transfer of the whole chromosome, rather than by an inverted or otherwise altered mode of transfer (7); this is very important for the late markers since such progeny could also arise through the formation of F′ factors in the donor population.

TABLE 1. *Strains employed*[a]

Strain	Genotype	Origin or reference
Hfr B4	*metB*λ⁻λ^R	2
Hfr B8	*metB*λ⁻λ^R	2
χ462	*proA leu metE lys trp purE lac str*	1
JC411	*leu met his argG str*	4
ED1195	*tyr argE his str*	Str^R (spontaneous) ex AT2273 (8)

[a] The nomenclature is that recommended by Taylor and Trotter (10).

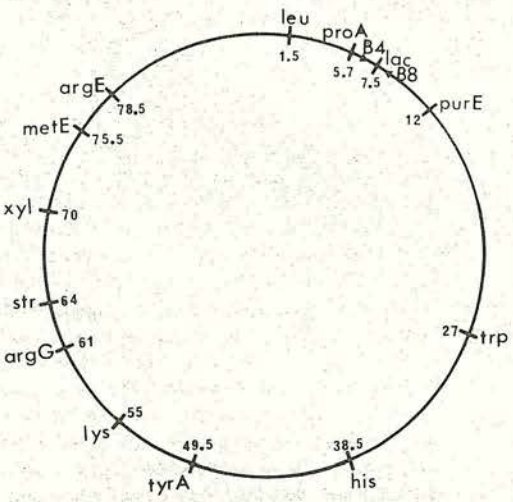

FIG. 1. *E. coli linkage map, with the origins of Hfr strains B4 and B8, and the positions of the markers used in this study. These are placed according to Taylor and Trotter (10) except that proA is moved from 6.5 to 5.7 min, and lac is moved from 9 to 7.5 min (3).*

Streptomycin was included in all plates. It did not appear that its presence altered the time of entry for markers transferred after the *str*⁺ allele.

Plates were incubated for at least 60 h; it was noticed that colonies arising from the transfer of later markers took longer to appear than those arising only from the transfer of earlier markers.

RESULTS

Interrupted matings were performed simultaneously between strain χ462 and Hfr strains B4 and B8, in flasks in the same shaking water bath. There was sufficient broth present to allow normal growth and conjugation throughout the duration of the mating experiments. With later markers, there are much lower yields of progeny. It was nevertheless possible to define adequate entry curves by plating as many samples as possible (generally every 1 or 2 min for any marker). Since in these crosses Lac⁺ progeny colonies were small and the yield of such colonies was variable, the data for *lac*⁺ were obtained by scoring PurE⁺ progeny of strain Hfr B4 and Pro⁺ or Leu⁺ progeny of strain Hfr B8, for the Lac⁺ phenotype. These gave t_E values for *lac*⁺ which were greater than those for *purE*⁺ and *pro*⁺; moreover, there were no Lac⁺ clones among earlier recombinant classes (e.g., Trp⁺ for strain B4 and MetE⁺ for strain B8). This is therefore a valid method for obtaining t_E values for *lac*⁺. The data from a pair of crosses (experiment number 4 in Table 2) are given in Fig. 2.

The t_E values inferred from the five pairs of crosses performed are presented in Table 2. Three minutes are deducted from all t_E values, since it has been shown (3) that this was the approximate "origin entry time" of these two strains. It appears that strain Hfr B4 transfers the chromosome in about 1.07 times the time taken by strain Hfr B8. We conclude that even sibling Hfr strains may transfer the chromosome at different rates. However, it should be noted that these strains arose from a common ancestor over ten years ago.

Perhaps the most important marker for this analysis other than *lac* is *lys*, which is situated about halfway round the chromosome from *lac*, at 55 min. Checks were made to show that the *lys*⁺ allele on the donor strains was not anomalous in being transposed or specified by two loci, for instance. Strains Hfr B4 and Hfr B8 were mated with strain JC411, which is ArgG⁻ His⁻, and strain ED1195, which is Tyr⁻ His⁻. *argG* and *tyr* lie on either side of *lys*, at 61 and 49.5 min, respectively. The t_E values obtained (Table 3) are consistent with these markers and *lys* being in their expected positions with respect to each other in strains B4 and B8.

The sum of the t_E values for any given marker from strains Hfr B4 and Hfr B8 represent the time taken to transfer DNA equal to one complete chromosome, since the two Hfr strains have closely located origins but opposite directions of transfer. This sum falls to a minimum for *lys* (Table 2); in other words, the first half of the chromosome is transferred by each Hfr strain faster than the second half. The speed of transfer does not appear to be determined by the specific segment of DNA transferred, but by the proximity of the DNA to the origin of transfer in each case. Deceleration in the rate of transfer of the chromosome in a mating pair has been proposed previously (5, 6; see 11). However, an alternative explanation is that not all donor cells transfer DNA at the same rate

TABLE 2. *t_E values (minutes) for markers in crosses between Hfr strains B4 and B8 and the F⁻ strain χ462*[a]

Donor	Expt no.	*proA*⁺	*leu*⁺	*metE*⁺	*lys*⁺	*trp*⁺	*purE*⁺	*lac*⁺
B4	1	1	6		51	107	134	139
	2	1	6.5		49	98	131	133
	3	1.5	6	28	58		129	141
	4	1	7.5	28	59	111	136	143
	5	1.5	7	28.5	55	98	128	133
	Avg	1.2	6.5	28	54.5	103.5	131.5	138
	% of t_E for *lac*	0.8	4.7	20	39.5	75	96	100
		purE⁺	*trp*⁺	*lys*⁺	*metE*⁺	*leu*⁺	*proA*⁺	*lac*⁺
B8	1	5.5		60		113	121	125
	2	5		60		120	127	131
	3	5.5	23	59	92	126	129	131
	4	5.5	23	58	94	125		132
	5	4.5	24	58	86	113		125
	Avg	5.2	23.5	59	91	119.5	126	129
	% of t_E for *lac*	4	18.2	46	70.5	93	97.5	100
Sum of t_E values for B4 and B8		136.7	127	113.5	119	126	127.2	

[a] The experiments are numbered to indicate concurrent matings. All the t_E values have had 3 min subtracted, to allow for the origin entry times determined by Broda (3). The ratios of the t_E values for *lac*⁺ transferred by Hfr strains B4 and B8 in the five experiments are 1.11, 1.02, 1.08, 1.08, 1.06, giving an average of 1.07.

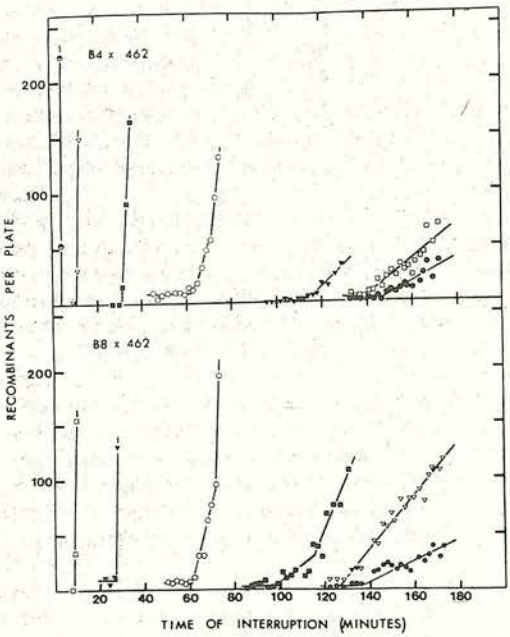

FIG. 2. *Plots of recombinant formation in concurrent crosses between strain χ462 and Hfr strains B4 and B8. The B4 × χ462 cross was started 1 min before the B8 × χ462 cross to obtain the t_E values given in Table 2, experiment 4; 3 minutes were subtracted from all values to allow for the origin entry times.*

during conjugation, and that the faster the rate of transfer, the greater the chance of the DNA breaking and transfer terminating. The recombinants for late markers would, therefore, arise from donors that transferred DNA more slowly than average, thus delaying the t_E from that expected from the chromosomal position of the late marker.

Whether the deceleration arises from the changing rate of transfer in each mating pair or from more complex properties of a heterogeneous population, it seemed worthwhile to characterize the process algebraically so that the behavior of the different Hfr strains could be compared.

For the ideal transfer process proceeding at a constant rate, the rate of transfer of DNA (x) during conjugation (dx/dt) equals a constant, k. If the process is decelerating, then this expression changes to include a negative term dependent in the simplest case on either x (model A) or t (model B).

Model A. If the deceleration were a function of the amount of DNA transferred (e.g., the donor cell is running out of energy or the recipient cell offers increasing resistance to the entry of the DNA) then the rate equation is $dx/dt = k - ax$, where a is a constant. This has an integral in the form: $k - ax = C\,e^{-at}$, where C is a constant. Subject to the boundary condition, $t = 0$

when $x = 0$, C must equal k, and the expression can be rewritten $x = k(1 - e^{-at})/a$. The second boundary condition used is that transfer of the *lac*$^+$ marker indicates complete transfer of the chromosome (i.e., $x = 90$ when $t = t_E$ for *lac*). This value of 90 min for total chromosome length is assumed in both models so as to fit with current usage, e.g., in the Taylor-Trotter map, but does not imply that this figure has any specific physical significance. The expression for the transfer of amount x DNA is now given as: $x = 90(1 - e^{-at})/(1 - e^{-at_{E\text{-}lac}})$. The value of a was obtained by computing the least-squares fit for the experimental data, and a was calculated to be 0.00412 for strain Hfr B4, and 0.00433 for strain Hfr B8.

Model B. If the deceleration is dependent on time (e.g., the cells change their condition steadily to become less able to transfer DNA) then the rate equation is given by $dx/dt = k - at$. This has the integral form $x = C + kt - at^2/2$. If $x = 0$ when $t = 0$, then C, the constant of integration, is 0. If we take as the second condition that undecelerated transfer would have taken 90 min, then $k = 1$. The value of a is now fixed by the observed time to transfer the total chromosome, which give values of a equal to 0.00252 for strain Hfr B4, and 0.002344 for strain Hfr B8.

In Table 4, the calculated t_M (map position)

TABLE 3. *t_E values for markers in crosses between Hfr strains B4 and B8 and F$^-$ strains JC411 and ED1195*[a]

Donor	Recipient	t_E values (min)					
		leu$^+$	*argE*$^+$	*xyl*$^+$	*argG*$^+$	*tyr*$^+$	*his*$^+$
B4	JC411	6.5			53		89
	ED1195		23	37		69	93
B8	JC411				69		38
	ED1195					54	37

[a] All t_E values have had 3 min subtracted.

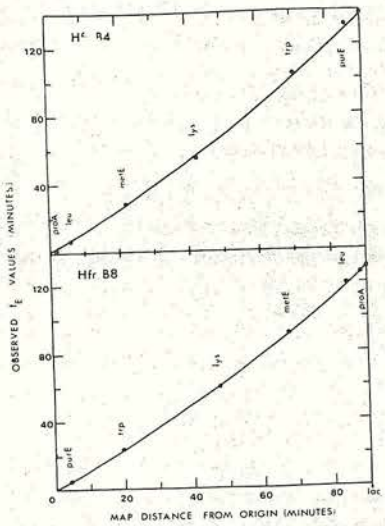

FIG. 3. *Mean observed t_E values in crosses between Hfr strains B4 and B8 and strain χ462, plotted against distance of markers from origin of chromosome transfer (see text). The curves are the calculated best-fit curves for these data, assuming deceleration according to model A (see text).*

TABLE 4. *Calculated mean distance of markers from origins of chromosome transfer of Hfr strains B4 and B8, based on the calculated best fit of all the observed t_E values given in Table 2 to model A*[a]

Donor (1)	Marker (2)	Map position[a] (t_M) (3)	Avg observed t_E (4)	Calculated t_M on model A (5)	3-5 (6)	Calculated t_M on model B (7)	3-7 (8)
Hfr B4	*proA*$^+$	1.8	1.2	1.0	+0.8	1.2	+0.6
	leu$^+$	6.0	6.5	5.6	+0.4	6.4	−0.4
	metE$^+$	22.0	28.0	22.8	−0.8	26.0	−4.0
	lys$^+$	42.5	54.5	41.7	+0.8	47.0	−4.5
	trp$^+$	70.5	103.5	72.1	−1.6	76.5	−6.0
	purE$^+$	85.5	131.5	86.9	−1.4	87.9	−2.4
	lac$^+$	90.0	138.0	90.0	0	90.0	0
Hfr B8	*purE*$^+$	4.5	5.2	4.7	−0.2	5.1	−0.6
	trp$^+$	19.5	23.5	20.2	−0.7	22.2	−2.7
	lys$^+$	47.5	59.0	47.5	0	50.8	−3.3
	metE$^+$	68.0	91.0	68.3	−0.3	71.6	−3.6
	leu$^+$	84.0	119.5	84.9	−0.9	86.0	−2.0
	proA$^+$	88.2	126.0	88.3	−0.1	88.8	−0.6
	lac$^+$	90.0	129.0	90.0	0	90.0	0

[a] Distance from origin of chromosome transfer, based on the Taylor-Trotter map (10), revised for *lac* and *proA* (3).

values for both models are presented, and those for model A are plotted in Fig. 3. *proA* and *lac* are placed at 5.7 and 7.5 min, respectively (3). In crosses with each Hfr strain, model A gives an excellent fit. The χ^2 value for the strain B4 data (which have 31 degrees of freedom) was 6.2. For the strain B8 data (29 degrees of freedom) it was 1.4. Both these values are highly significant. Model B generates less well-fitting curves, and for both Hfr strains the observed t_E values correspond to calculated t_M values which are consistently less that those expected from the Taylor-Trotter map. The systematic nature of these discrepancies shows that model B cannot account adequately for the physical characteristics of the transfer process.

DISCUSSION

Since the physical basis of the Taylor-Trotter genetic map is a single DNA molecule in which the genes have fixed relationships to each other, the observation of nonlinear patterns of transfer by both the Hfr strains cannot have its origin in any inaccuracy in the map, but must arise as a consequence of the transfer process itself having nonlinear properties. The data presented in Table 2 also show that two sibling Hfr strains transfer the whole chromosome at different and decelerating rates. Therefore, one cannot, without qualification, directly compare transfer times for markers at different distances from the origin of a given Hfr or times obtained with different Hfr strains.

The problem of obtaining t_E values from plots of recombinant formation against time has been mentioned in the preceding paper (3). The method used here, which uses the very early recombinants, probably overestimates t_E values for late markers since with these markers the first recombinants to be observed represent a larger proportion of the total recombinant population. This would make the true deceleration in transfer somewhat less than the apparent deceleration.

The computed best-fit curves with model A are surprisingly good. If, therefore, deceleration does occur as a linear function of the amount of chromosome already transferred, the markers examined here are very accurately placed relative to each other on the Taylor-Trotter map using the revised positions of *proA* (5.7 min) and *lac* (7.5 min) given in the previous paper (3). Since the scatter of points about the curves is small and, on comparison of the data for Hfr strains B4 and B8, it is random, there is no case for moving any of the other markers to new positions. It is of interest to note that on model A the segments 0 to 10, 10 to 20, 20 to 30, and 30 to 40 min will be transferred by Hfr strain B8 in 11.25, 11.83, 12.47, and 13.18 min, respectively. However, we can only equate corrected transfer distance with physical distance (i.e., the number of bases transferred) if we assume that there are no chromosomal regions which have the intrinsic property of being transferred faster or slower than other regions. Discrete regions behaving anomalously should, however, be detected in experiments with paired Hfr strains such as B4 and B8, as localized regions of poor fit, with one or both Hfr crosses.

These experiments underline the need for caution when comparing and compiling segments of the genetic map. The first two assumptions listed above—that different Hfr strains transfer the chromosome at similar rates, and that a given Hfr strain transfers markers at time intervals proportional to the distance apart—are clearly incorrect. Although the difference in transfer rate, about 7%, between the two Hfr strains, is small, it is believed to be real from the consistency of the trend observed in the five pairs of crosses.

The non-linearity of the entry times for different markers when plotted against map distances based on the Taylor-Trotter map is quite pronounced with both Hfr strains. The form of the curve fits in each case a similar decelerating function (model A), as though the origin of this behavior is some property of the transfer process itself or of the recipient used. Possible variations in behavior between different recipient strains—a test of the third assumption given above—have not been examined here.

With regard to the fourth assumption, that data from different laboratories should give comparable entry times, it is likely that the improvement and standardization of techniques may increase agreement, although the experimental results shown in Table 2 contain more variation than one would ideally like to see. Since in each experiment there is a tendency for all the t_E values to be earlier or later than the average, slight variations in the physiological state of the mating cells or of the conditions of mating influence the gross transfer rates and limit the absolute accuracy which can be reached at the present.

The fact that model A, showing deceleration with amount of DNA transferred, clearly gives the better fit to the experimental data cannot be taken to define the physical basis of the deceleration, though two possibilities—limitation of energy sources or packing problems in the recipient—have been mentioned. In particular, it has not been shown whether the deceleration process is a property of each individual mating

or whether it arises from heterogeneity in the transfer behavior of different donor cells. Further experiments are needed to clarify this aspect. However, model A does have good predictive value for transfer times and chromosome distances even though at present it represents only an empirical relationship.

ACKNOWLEDGMENT

We thank Rosemary Pringle for her excellent assistance.

LITERATURE CITED

1. Berg, C. M., and R. Curtiss III. 1967. Transposition derivatives of an Hfr strain of *Escherichia coli* K12. Genetics **56**:503-525.
2. Broda, P. 1967. The formation of Hfr strains in *Escherichia coli* K12. Genet. Res. Camb. **9**:35-47.
3. Broda, P. 1974. Modified map positions for *lac* and the *pro* markers in *Escherichia coli* K-12. J. Bacteriol. **117**:741-746.
4. Clark, A. J., and A. D. Margulies. 1965. Isolation and characterization of recombination-deficient mutants of *E. coli* K12. Proc. Nat. Acad. Sci. U.S.A. **53**:451-459.
5. Fulton, C. 1965. Continuous chromosome transfer in *Escherichia coli*. Genetics **52**:55-74.
6. Jacob, F., and E. L. Wollman. 1958. Genetic and physical determinations of chromosomal segments in *Escherichia coli*. Symp. Soc. Exp. Biol. **12**:75-92.
7. Low, B. 1967. Inversion of transfer modes and sex factor-chromosome interactions in conjugation in *Escherichia coli*. J. Bacteriol. **93**:98-106.
8. Masters, M., and P. Broda. 1971. Evidence for the bidirectional replication of the *Escherichia coli* chromosome. Nature N. Biol. **232**:137-140.
9. Taylor, A. L., and M. S. Thoman. 1964. The genetic map of *Escherichia coli* K12. Genetics **50**:659-677.
10. Taylor, A. L., and C. D. Trotter. 1972. Linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **36**:504-524.
11. Wood, T. H. 1968. Effects of temperature, agitation, and donor strain on chromosome transfer in *Escherichia coli* K-12. J. Bacteriol. **96**:2077-2084.

(Reprinted from Nature, Vol. 258, No. 5537, pp. 722–723, December 25, 1975)

otility, diffusion and cell concentration fect pair formation in *Escherichia coli*

the high cell densities normally used in *E.coli* matings out 10^8 cells per ml) individual cells undergo frequent and eated collisions, and should therefore have multiple opportities for pairing[1–3]. A mating pair of cells, once formed, will ilarly continue to collide almost as frequently with other s. It is not known whether these secondary collisions affect ıer the speed or extent of DNA transfer, since the true perties of a mating pair of cells formed with little likelihood urther collisions have not been studied. Pairs formed at low concentrations, however, should be suitable for such dies; indeed, they could be regarded as paradigms of simple –cell interactions. We show here that at low densities pair mation is much more efficient than at high densities, and) that motility decreases this efficiency once cells have ided.

t was necessary to estimate both the number of collisions ween cells and the number of pairs formed. Cell movement observed directly and the formation of progeny was used n index of pairs formed. Since shaking introduces additional tive cell movement[4], all matings were carried out without king.

n the low density matings, inputs of both donor and recipient s of about 10^5 per ml gave well defined progeny counts and wed exponential growth of both parents during 1 h. The cted marker, *purE*$^+$, enters the recipient cell approximately ıin after DNA transfer starts[5]. The formation of progeny the viable counts during such an experiment using non-tile strains are shown in Fig. 1. The rate of formation of geny accelerates steadily, and their number does not exceed % of either parent during the experiment. The lag in the earance of progeny strongly suggests that an unstable ring which is sensitive to the experimental handling conditions cedes the more stable pairing which survives the pipetting mild vortexing used. This suggests the model

$$D+R \rightleftharpoons D\text{–}R_{unstable} \longrightarrow D\text{–}R_{stable}$$

ere D represents donors, R recipients and $D\text{–}R_{stable}$ the nplexes which give rise to progeny colonies. For practical rposes, however, we have estimated a minimum value for rate constant from the simplified model

$$D+R \xrightarrow{k} D\text{–}R_{stable}$$

uming a time lag for the conversion of any pair into a stable r of about 9 min, as the data suggest.

The experimental data from low density matings could be fitted to the simplified model at early times but the numbers of progeny found during the later stages of the experiment consistently exceeded expectation. We have observed under the microscope that both cells in apparent mating pairs, when placed on agar blocks and incubated further, continue to divide. We have therefore assumed that the number of progeny cells (P) can increase due to multiplication at a rate characteristic of the recipient strain. The equations used to model the process were

$$dP/dT = kDR + k_R P$$
$$dD/dT = k_D D$$
$$dR/dT = k_R R$$

where k_D and k_R are the observed growth rate constants for donor and recipient respectively. The theoretical numbers of progeny were computed using the cell numbers at the time of mixing as the initial conditions. The value of the second order rate constant, k, used to generate the theoretical curve (which incorporates the lag previously mentioned) shown in Fig. 1 was 7×10^8 l per 6×10^{23} cells per s. These units facilitate comparison of the rate constant with the upper limit for a second order rate constant in liquids for a reaction between neutral molecules, 3×10^9 l mol^{-1} s^{-1}, in a diffusion-limited reaction[9]. Pairing is therefore an inherently efficient process following the initial contact, as suggested by Nelson[10], particularly since not all cells in the population may be competent to mate[4,10] and some mating pairs will fail to produce recombinants for the selected marker[11].

The apparent rate constant for the mating (at low density) between motile parental strains is, as expected, significantly greater than for non-motile strains (Table 1). The apparent rate constants for the two crosses involving one motile and one non-motile parent, which might be expected to be similar, show an interesting asymmetry; the cross between a motile donor strain and a non-motile recipient strain is more fertile than the complementary cross. But the significance of the difference between the first two crosses mentioned, and of the asymmetry in the latter two crosses, can be evaluated only if the contribution of cell movement to the collision rate is known.

To this end, we included in these experiments measurements of cell movement. During each mating, videotape records were made of microscopic fields of samples from the parental cultures, observed in a closed chamber at 37 °C, while the bulk cultures were mated. The recordings were replayed to obtain tracings of the tracks of individual cells for the calculation of apparent diffusion coefficients. The relative magnitudes of the upper limits for the rate constants are directly proportional to the sum of the apparent diffusion coefficients for donor and recipients; we assume that the cell–cell distance on contact

Table 1 Effect of motility and concentration on the relative efficiency of mating pair formation

Mean parental cell numbers per ml	Motility of strains Donor	Recipient	Relative mutual diffusion coefficients	Observed rate constant $\times10^{-10}$ (l per 6×10^{23} cells per s)	Relative efficiency of pair formation
1.6×10^5	–	–	1	0.07	1
	+	+	19.8	0.6	0.43
	+	–	9.3	0.6	0.92
	–	+	11.4	0.09	0.11
3.2×10^6	–	–	1	0.1	1.43
	+	+	48.8	1.3	0.38
3.6×10^8	–	–	0.8	0.006	0.11
	+	+	4.0	0.0015	0.0054

hree sets of matings were carried out; within each set there was an approximate 1:1 ratio of donor to recipient cells, and a similar total input. The donor strains were B8 (motile) and ED1064 (non-motile); recipient strains were χ478 (motile) and ED1065 (non-motile). The ues of the relative mutual diffusion coefficients and of the relative efficiencies of pair formation were chosen to be 1 for the cross between -motile parental strains at the lowest concentration.

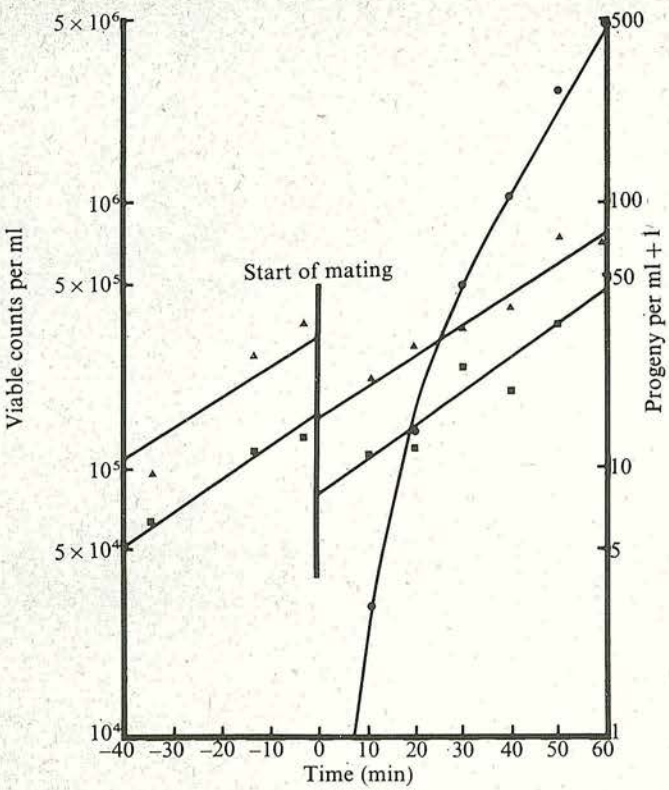

Fig. 1 Progress of the mating between the *E. coli* strains ED1064 and ED1065. These were isolated as spontaneously arising non-motile derivatives from, respectively, cells of Hfr B8 (refs 5 and 6; *metB*) and χ478 (ref. 7; F⁻*proC purE trp lys metE leu lac str*) that had survived a challenge from bacteriophage χ (provided by E. Meynell), which only attacks motile cells[8]. Cultures were grown in L broth at 37 °C to about 2×10^8 cells per ml, diluted to about 10^4 cells per ml in fresh L broth and grown for a further 90 min to approximately 2×10^5 cells per ml in a rotary shaking water bath (100 r.p.m.). Equal volumes of donor and recipient cultures were then mixed; incubation continued without shaking. Separate samples were diluted as appropriate in buffer, vortexed gently and pipetted into molten agar held at 46 °C; these samples were then poured on to different supplemented minimal agars selective for Pur⁺ (Str^r) progeny and also for each parental strain. Viable counts per ml of donor (▲) and recipient (■) and the number of progeny per ml +1 (●) are shown semi-logarithmically with the curve of the predicted progeny per ml+1, assuming that $k = 7 \times 10^{-8}$ l per 6×10^{23} cells per s.

is the same in all crosses. From the ratio of the observed rate constants to the relative upper limits in these matings, we can calculate the relative efficiencies of pair formation per collision (Table 1).

The most efficient matings are those between the two non-motile strains at the lower densities. The corresponding crosses between the motile strains B8 and χ478 are about one-third as efficient. Since the average interval between successive collisions involving a given cell at the lowest concentration exceeds the duration of the experiment, this lower efficiency is an inherent property of mating pairs of motile cells.

The relative efficiencies of the matings involving one motile and one non-motile parent show that the asymmetry noted above is not due to different mobilities of strains B8 and χ478. Therefore other possibilities, including vectorial movement by the donor strain, must be considered.

Cell concentration also affected the relative efficiency. Thus, when one allows for a tenfold drop in the diffusion coefficient of the motile cells (but not of the non-motile cells) at the highest concentration, the crosses between motile cells and between non-motile cells were both ten times less efficient. These low efficiencies may be due to the additional collisions, which could result in disruption of existing pairs or the addition of further cells to existing complexes. We therefore suggest that physiological and genetic analysis of low and moderate density matings will be the most reliable source of information about the behaviour and properties of a simple mating pair of cells.

We thank Helen Grozier and Clive Duggleby for their assistance.

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Received July 31; accepted November 13, 1975.

1 Fischer-Fantuzzi, L., and di Girolamo, M., *Genetics*, **46**, 1305–1315 (1961).
2 Ou, J. T., *J. Bact.*, **111**, 177–185 (1972).
3 Achtman, M., *J. Bact.*, **123**, 505–515 (1975).
4 Walmsley, R. H., *J. Bact.*, **114**, 144–151 (1973).
5 Broda, P., *J. Bact.*, **117**, 741–746 (1974).
6 Broda, P., *Genet. Res., Camb.*, **9**, 35–47 (1967).
7 Berg, C. M., and Curtiss, R., *Genetics*, **56**, 503–525 (1967).
8 Meynell, E., *J. gen. Microbiol.*, **25**, 253–290 (1961).
9 Amdur, I., and Hammes, G. G., *Chemical Kinetics* (McGraw-Hill, New York, 1966).
10 Nelson, T. C., *J. cell. comp. Physiol.*, **48**, 271–291 (1956).
11 Jacob, F., and Wollman, E. L., *Sexuality and the Genetics of Bacteria* (Academic, London, 1961).

Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

t. Res., Camb. (1978), 31, pp. 167–175
ted in Great Britain

Role of simple and complex aggregates in *Escherichia coli* Hfr × F⁻ matings

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(*Received* 4 *October* 1977)

SUMMARY

Analysis of tetraparental Hfr × F⁻ matings of *Escherichia coli* strains showed that mating complexes were predominantly pairs or small aggregates of cells. Many physically associated complexes of donor and recipient cells gave rise to no recombinant cells. The observed linkage of genetic markers in recombinants is sensitive to multiple mating events, and should therefore be measured under well-defined conditions giving pairwise matings only.

1. INTRODUCTION

ne mating process in *Escherichia coli* between Hfr or F′ donor cells and ecipient cells is interesting as a model of cell–cell interactions. We have shown the efficiency with which recombinants are formed varies over a considerable e, and depends on cell concentration and on cell mobility (Collins & Broda,). At high concentrations, the process is complicated by the fact that collisions rther cells with the primary mating complex may either disrupt such pairs sult in the formation of more complex mating aggregates, perhaps of the reported by Fischer-Fantuzzi & di Girolamo (1965). Achtman (1975) has mented the existence of large aggregates of cells in mating mixes using cle size determinations with a Coulter Counter and microscopy. Since multiple egates might allow multiple transfer, they could be one cause of the genetic plexity observed in female exconjugants in some matings (Bresler, Lanzov & ukian, 1967, 1973; Lotan, Yagil & Bracha, 1972). However, even single le exconjugants obtained by micromanipulation have been observed to give to a number of different classes of progeny (Lederberg, 1957; Anderson, ; Wood, 1967).

some stage the process of aggregation must be limited by factors such as utilization of all the potential for contact formation afforded by the pili. inly aggregates do not form as fast as expected on the basis of the kinetics ll collisions (Collins & Broda, 1975); indeed, some cells appear not to partici- at all in mating (Nelson, 1956; Walmsley, 1973).

e have attempted to answer the following questions about a mating between ally growing cells:

what proportion of mating cells are in complex aggregates;

(ii) what proportions of the recombinant cells arise in complex and sim aggregates, respectively;

(iii) what differences can be observed between the patterns of recombina classes arising in colonies derived from simple and complex mating aggregat respectively?

We have used two genetically distinguishable donor strains with two genetica distinguishable recipient strains in a four-parental mating. At intervals, samp were diluted and plated on nutrient agar and agar selective for recombina colonies. By determining the incidence of the different parental types in a numl of colonies, it was possible to calculate the proportion of colonies that der from more than one donor cell or more than one recipient cell; that is, of th formed from complex mating aggregates. We find that about 30% of don recipient complexes give rise to recombinants. Further, only a minority of mat complexes involve more than a single donor cell and a single recipient cell. T pattern of progeny classes in all cases could have arisen through a small num of independent recombination events.

2. MATERIALS AND METHODS

(*a*) *Strains.* The donor strains of *E. coli* used were Hfr B8, which is *metB lac*$^+$ (Broda, 1967), and a spontaneously arising Lac$^-$ derivative, ED3688. In int rupted mating experiments, the times of entry of the markers relevant h were: *proC*$^+$ 4 min; *purE*$^+$ 8 min; and *trp*$^+$ 26 min; *lac*$^+$ is transferred very l (Broda, 1974; Broda & Collins, 1974). The recipient strains were X478 which *proC purE trp lys metE leu lac str* (Berg & Curtiss, 1947) and its Lac$^-$ derivat ED1199, made by P1 transduction.

(*b*) *Latex particles.* The calibration P.V.T. latex suspension (Coulter Electron Ltd, Harpenden) contained particles with a diameter of 2·03 μm.

(*c*) *Matings.* The fertility of the Hfr strains was checked beforehand by test 100 patched colonies with the recipient strains in plate matings. The proport of fertile colonies ranged from 98 to 100%.

Cultures for mating were grown in L-broth with moderate shaking. Mat cultures were not shaken further. All dilutions were performed with L-br using 1 ml pipettes to minimize shear. Blending and other procedures w performed as previously described (Broda, 1974).

(*d*) *Direct observation of aggregates.* Samples of mating cultures were fixed formaldehyde and plated on agar directly, for microscopic examination photography. The cells were not distributed evenly in the microscopic fields could be found in local concentrations, possibly due to channelling effects in preparation of the slides. Additional samples prepared by the method of Achtn (1975) on gelatin slides showed the same phenomenon. It was considered import to use an internal control for these observations, which were therefore repeated w mating cultures to which a suspension of latex particles had been added. Sam were taken and plated as before, and also after a tenfold dilution in the fixat

clumps of cells were noted in the undiluted samples on both agar and in, but so too were clumps of latex particles, and of cells and latex particles her. In the samples diluted tenfold before plating, fewer aggregates of cells of latex were observed. Since mating aggregates are known to be resistant lution, the aggregates observed in undiluted samples must have included artifacts. Therefore microscopy can only be used to obtain qualitative mation on the complexity of mating.

Limitations on DNA transfer after challenge with streptomycin. A different oach involving tetraparental matings was adopted because it was free of difficulties and also gave information on the events leading to recom- t formation. For this analysis it was necessary to establish conditions where ling from the mating culture did not interfere with the formation of and Trp$^+$ recombinants by existing mating complexes. Otherwise the eritance of *trp*$^+$ among Pur$^+$ progeny would be a function of the duration of ng before plating. We therefore determined how much residual transfer from 38 to strain X478 was possible after (A) dilution into broth containing tomycin, and holding for 30 min before plating, and (B) dilution followed rect plating onto selective agar. The yields from samples taken at intervals reated thus were compared to those obtained from two parallel samples d D) which were blended prior to dilution but otherwise treated as samples l B.

both Pur and Trp the data from the unblended samples (A and B) were similar to each other. The data from the blended samples (C and D) also bled each other but showed a characteristic displacement from the A and ues. This was more pronounced for the Trp marker, and is an index of the on on the chromosome relative to the origin of transfer. This result with D implies that once recipient cells have received the DNA for the nutritional er their ability to integrate it is approximately the same in broth and on lective agar. The similarity between the A and B results then implies that the cells have been challenged with streptomycin, the same amount of al DNA transfer is possible in broth or on selective agar. As the numbers p$^+$ progeny rose during the course of the experiment relative to the number r$^+$ progeny, we conclude that streptomycin limits Trp$^+$ DNA transfer to ater extent than it limits Pur$^+$ DNA transfer. The introduction of strepto- immediately after sampling does not allow us to see how the mating gates present at the time of sampling would have behaved if they had been ndisturbed. We have therefore chosen to study the properties of mating gates by diluting the samples to prevent new collisions and holding for without streptomycin to allow transfer of *trp*$^+$ to be accomplished before g.

Tetraparental matings at two cell concentrations. Exponentially growing cul- of the two donor strains were mixed to give approximately equal numbers s. The same was done with the two recipient strains. One generation later, samples of the mixed donor and of the mixed recipient cultures were

combined (A) and mating proceeded without shaking. Ten min after the st of the A mating, another mating mixture (b) was established using 0·7 ml e of the mixed donor and the mixed recipient cultures, and 18 ml of prewarr broth, giving one-tenth the cell concentration of the first mating. Other sam were diluted appropriately (at least $\times 10^4$ for cross A and $\times 10^3$ for cross Samples were held in broth for 30 min; portions of these samples were then pla onto selective plates for Pur$^+$ progeny without further dilution and, after dilut on nutrient plates.

(*g*) *Colony analysis.* Multiple platings were employed in order to prod enough colonies for further analysis. With 50 or fewer colonies per plate, chances of coincidental platings of cells not physically associated together calculated to be less than one per plate (assuming that cells closer than 2 might develop into contiguous colonies that could be mistaken for a sir colony). Whole colonies from either nutrient plates or from recombinant-selec plates were emulsified in 5 ml of buffer. Samples of 0·1 ml from dilution 10^4-fold were spread onto the appropriate plates: minimal supplemented v methionine to allow growth of the donor strains only, minimal with appropr supplements plus streptomycin to allow growth of the recipient strain or spe recombinant classes. A mean of 100 colonies per plate was obtained. Such pl were replicated after 2 days onto further media including lactose MacCon plates to identify individual cell types. Since these operations took consider time, it was necessary to show that there was no selective death within colonies themselves; no changes in the patterns were detected from sam analysed at intervals of up to 2 weeks.

3. RESULTS

In view of the difficulties encountered with the direct method of visualiza of mating aggregates (see Materials and Methods), we employed the less di tetraparental mating method. We first showed that after dilution to pre further aggregate formation, a holding period of 30 min sufficed to allow tran of DNA to the last marker, *trp*, being observed in the recombinants (see Mate and Methods). We could therefore compare the behaviour of mating aggreg sampled from the mating culture at different times.

(i) *The tetraparental cross*

The yields of viable colonies on both nutrient and selective plates in the crosses are given in Table 1. In cross *A*, the yield of Pur$^+$ recombinants rise about 5% of the recipient colony count, and in cross *B* it reaches about 1· Therefore the yields in the two crosses are not in proportion to the produc the donor and recipient cell concentrations (a ratio of 100:1 would have expected, on the basis of Hayes (1957)). Instead the efficiency drastically dec at the higher cell concentration, as previously reported (Collins & Broda, 1 Achtman, 1975).

(ii) *Complexity of mating aggregates*

lonies from nutrient plates from the 50 min samples (which might be expected ntain the most complex aggregates) were emulsified and analysed for their n composition. Only 8% and 5% of the 300 colonies examined from the d B crosses respectively were found to contain both donor and recipient Hence such mating aggregates account for only the minority of the cells

e 1. *Pur+ recombinant formation and colony-forming units on broth agar in parent mating (per ml of mating cultures). In cross A, the initial cell concentration c. tenfold that in cross B*

	Time of initial dilution (min)					
	10	15	30	35	50	55
Pur+ recombinants ($\times 10^{-7}$)	0·41	0·77	1·4	1·7	2·2	1·8
Donors ($\times 10^{-8}$)	1·7	—	2·3	—	2·8	—
Recipients ($\times 10^{-8}$)	2·6	—	3·6	—	4·3	—
Ratio: $\frac{\text{Recombinants}}{\text{Recipients}}$	0·016	—	0·039	—	0·051	—
Pur+ recombinants ($\times 10^{-6}$)	0·1	0·2	0·6	0·5	0·8	1·5
Donors ($\times 10^{-8}$)	1·4	—	2·5	—	3·9	—
Recipients ($\times 10^{-8}$)	1·9	—	3·2	—	5·0	—
Ratio: $\frac{\text{Recombinants}}{\text{Recipients}}$	0·005	—	0·019	—	0·016	—

th crosses at this time. Mating complexes are stable during dilution, holding plating (see below). However, during the 30 min holding period the number cipient cells increases by a factor of 1·4 (data not shown). Therefore, the nd 8·5% of the recipients in the A and B crosses that were engaged in tial mating aggregates at the time of plating correspond to values of 16·9 2% respectively at the time of dilution.

wever, it was possible that some female exconjugants were by this time in) free of donor cells, so that the number of mixed colonies was an under- ate of the number of cells participating in matings. Therefore, as well as s from all colonies that contained both donor cells and recipient cells, clones some colonies derived from apparently uncomplexed female cells were also d for parental types and recombinant classes present (Table 2).

ong the recipient cells apparently complexed with donors, only about 30% e A cross) and 20% (in the B cross) gave evidence of recombinant formation; is, at least two-thirds of the mixed colonies gave none. Therefore many ng aggregates' or pairs may be non-productive. This result also means that ounds of mating in the growing colony are rare. In contrast, only one of uncomplexed recipient cells (from the A cross) and only one of fifty from cross contained recombinant cells. This result suggests that most recipient involved in successful matings remain complexed with donor cells, even

though in many such pairs DNA transfer will have stopped. Therefore experimental procedure is an assay of past and present mating complexes.

Only one colony (from the A cross) contained both Lac+ and Lac− recipient c Therefore, allowing for the equal number of colonies expected to have ar either from two Lac+ or two Lac− recipient cells, we can calculate that

Table 2. *Comparison of the genotype classes present in F− clones isolated f individual colonies on viable count plates for the 50 min samples (cf. Table Colonies were tested by replicating for the Pro, Pur and Trp phenotypes*

	Cross	Recipient genotype only	Recombinant: Single class	Recombinant: Mixed classes	Recipient + Recombinant: Single class	Recipient + Recombinant: Mixed classes	Lac+	T
(i) From mixed donor–recipient colonies	A	15	1	2	1	4	14	
	B	12	1	0	1	1	8	
(ii) From apparently unassociated recipients.	A	59	0	0	1	0	30	
	B	49	0	0	1	0	26	

about 10% of the aggregates should contain two recipient cells. Insufficient were available to make any conclusions about the number of donor cells mating aggregate.

Only one among the apparently uncomplexed recipient clones tested, none of the apparently uncomplexed donor clones tested was mixed with res to the Lac phenotype. There must therefore be only few accidental mixed colo (i.e. formed by coincidence on the plate).

(iii) *Complexity of recombinant colonies*

We have also assessed the complexity of Pur+ recombinant colonies ari in selective medium after sampling at 10, 30 and 50 min in the same two mat Such clones were emulsified and plated as described in Methods. In the c where the clones were mixed with respect to recombinant classes the rela proportions of the different classes were often quite different, though no system bias towards particular classes was observed.

The numbers of recombinant colonies that contained Lac+ and Lac− cel the A cross were 6/94 (10 min), 9/94 (30 min) and 17/94 (50 min). The c sponding values for the B cross were 1/80, 1/93 and 2/94. Therefore with there is a slight trend towards complexity. However, the pattern of inherit of donor markers did not change significantly, and for this reason we pre the pooled results from the A and B crosses in Table 3.

The majority of clones are pure in the sense that from each such clone colonies with a single Pro and a single Trp phenotype were observed. This most readily be understood if such matings are between a single donor a single recipient and involve a single round of recombination. The geneti

ed clones could then result from matings involving more than one recipient or from recipient cells (or colonies) in which more than one round of recombination had occurred. We can assess the frequency of the first type of event by ysing Pur+ colonies that contain both Lac+ and Lac− colonies. Of 32 such nies in cross A, over half were mixed with respect to the donor markers. he assumption that the most common complex aggregate has only two recipient , an equal number would have remained undetected because both recipient

Table 3. *The coinheritance of unselected markers among Pur+ recombinants of the A and B crosses*

ensity mating cross B)	Colonies with single recombinant class	Recombinant classes found				Colonies with more than one recombinant class
		Pro+ Trp+	Pro+ Trp−	Pro− Trp+	Pro− Trp−	
nies analysed	226	4	6	21	195	40
onies from I ing both Lac+ c− recipient colonies)	1	0	0	0	1	3
rrected data olonies believed e arisen from mating pairs II) = 258 s	224 (86·8 %)	4 (1·5 %)	6 (2·3 %)	21 (8·1 %)	193 (74·8 %)	34 (13·2 %)
nsity mating A)						
nies analysed	219	3	14	26	176	63
onies from I ing both Lac+ c− recipient 2 colonies)	14	0	0	0	14	18
rrected data olonies believed arisen from mating pairs II) = 218 s	191 (87·6 %)	3 (1·4 %)	14 (6·4 %)	26 (11·9 %)	148 (67·9 %)	27 (12·4 %)

were Lac+ or Lac−. After making allowance for such undetected complexes, ound that the remaining Pur+ recombinant clones, presumably derived from le mating pairs, were mainly genetically pure.

e data from the B cross show a simpler picture – fewer complex mating gates, arising slowly during the experiment, and simpler patterns of reinant classes present in the colonies analysed. We can again deduce the viour of simple mating pairs (Table 3). If these deductions are valid, then utcome of simple pairwise matings deduced from the data from each cross d be similar; the agreement is considered good.

4. DISCUSSION

We have adopted the tetraparental mating technique to assess the comple of matings between exponentially growing cells of two well-characterized stra Hfr B8 and X478. The mating complexes are unlikely to have been disru mechanically, and transfer between cells can continue first in broth and the solid agar. Our analysis is directed at the commonest events; that is, what is composition of the major classes of mating complex, and what is the composi of the recombinant colonies derived therefrom? We can only detect mino classes down to about 1% of the total number of recombinant cells.

For the A and B crosses, the consistent patterns of inheritance of unsele markers among the Pur^+ recombinants at the different sampling times estab that our manipulations do not interfere with zygosis. The absence of free combinants, even after the sampling manipulations and the 30 min perio holding in broth, confirms the stability of the mating complexes even after D transfer may have ceased.

We find that even in the A cross, the predominant class of mating com contains a single donor and a single recipient cell. Multiple matings can ind the demonstrated, but probably have a significant role only late in the mat The B cross contains even fewer multiparental matings and approximates to ideal condition of mating, i.e. where all complexes are simple pairs. Thi relevant to our consideration of progeny analyses.

Perhaps surprisingly, most presumptive mating complexes (i.e. mixed don recipient colonies recovered from nutrient plates) showed no evidence that gen transfer had occurred, so that few if any rounds of mating and/or recombina take place in the colony itself within the first few generations after plating. reason for this is unclear. However, the main implication here is that the recon nant cells found have descended from one or a few recipient cells in each colo Post-zygotic events are therefore predominantly simple. In cross A, the pres of complex aggregates raises markedly the proportion of colonies contai mixed recombinant classes, and the proportion of colonies with a single recon nant class (particularly one of the less common ones) fall significantly.

The properties of the simple pair that does produce progeny have been dedu for crosses A and B in Table 3. Eighty-seven per cent or more show a si recombinant class observed among the *c.* 100 single cell descendants of the orig zygote that could be examined by our methods. The role of multiple round recombination observed when HfrH was the donor (Anderson, 1958) but m less with HfrC (Lederberg, 1957) is therefore minimal in this system. In cont the majority of colonies in crosses A and B known to be derived by tran into the two distinguishable recipient strains contain multiple classes of reco nant (Table 3). However, the complexity of these mixed colonies was me that expected on the assumption that in each colony two independent sin matings with the properties given in Table 3 had occurred. Complex mat raise the observed frequency of any recombinant class relative to the num

lonies observed, but this change is much more pronounced in the case of nor recombinant class such as *pur*$^+$ *pro*$^+$ *trp*$^+$. The data deduced for 218 le mating pairs in cross A show that the class *pur*$^+$ *pro*$^+$ *trp*$^+$ occurred three in 191 colonies containing a single recombinant class and three times in olonies containing more than one recombinant class, i.e. six times in 218 (2·75 %). In the 32 colonies derived from aggregates that contained zygotes both recipient strains in cross A, the class *pur*$^+$ *pro*$^+$ *trp*$^+$ occurred four (12·5 %). The use of mating conditions in which complex aggregates are common will therefore lead to linkage data which differ even more from obtained under the more ideal conditions afforded by low density matings hich all recombinants are derived from simple mating pairs.

thank Helen Grozier for excellent assistance, and the Medical Research Council for rt.

REFERENCES

MAN, M. (1975). Mating aggregates in *Escherichia coli* conjugation. *Journal of Bacteriology* , 505–515.

RSON, T. F. (1958). Recombination and segregation in *E. coli*. *Cold Spring Harbor mposia on Quantitative Biology* **23**, 47–58.

, C. M. & CURTISS, R. (1967). Transposition derivatives of an Hfr strain of *Escherichia* K12. *Genetics* **56**, 503–525.

LER, S. E., LANZOV, V. A. & MANUKIAN, L. R. (1973). Mechanism of genetic recombi- ion during bacterial conjugation of *Escherichia coli* K12. IV. Heterogeneity of progeny xconjugants. Role of donor and recipient strains. *Molecular and general Genetics* **123**, –353.

A, P. (1967). The formation of Hfr strains in *Escherichia coli* K12. *Genetical Research* 5–47.

A, P. (1974). Modified map positions for *lac* and the *pro* markers in *Escherichia coli* 2. *Journal of Bacteriology* **117**, 741–746.

A, P. & COLLINS, J. F. (1974). Gross map distances and Hfr transfer times in *Escherichia* K12. *Journal of Bacteriology* **117**, 747–752.

NS, J. F. & BRODA, P. (1975). Motility, diffusion and cell concentration affect pair nation in *Escherichia coli*. *Nature* **258**, 722–723.

ER-FANTUZZI, L. & DI GIROLAMO, M. (1965). Triparental matings in *E. coli Genetics* 1305–1315.

S, W. (1957). The kinetics of the mating process in *E. coli*. *Journal of General Micro- ogy* **16**, 97–119.

RBERG, J. (1957). Sibling recombinants in zygote pedigrees of *E. coli*. *Proceedings of National Academy of Sciences, U.S.A.* **43**, 1060–1065.

, D., YAGIL, E. & BRACHA, M. (1972). Bacterial conjugation: an analysis of mixed mbinant clones. *Genetics* **72**, 381–391.

N, T. C. (1956). Sexual competence in *Escherichia coli*. *Journal of Cellular and Com- ative Physiology* **48**, 271–291.

SLEY, R. H. (1973). Physical assay for competence for specific mating pair formation . *coli*. *Journal of Bacteriology* **114**, 144–151.

, T. H. (1967). Genetic recombination in *Escherichia coli*: clone heterogeneity and the etics of segregation. *Science* **157**, 319–321.

Molec. gen. Genet. 138, 65—69 (1975)

Transience of the Donor State in an *Escherichia coli* K12 Strain Carrying a Repressed R Factor

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Received January 27, 1975

Summary. De-repression of the plasmid R100 in *Escherichia coli* is essentially a transient phenomenon resulting in the transfer of several R factors to different recipient cells from a single donor cell.

Strains carrying typical R factors succeed in transferring them to suitable recipient strains only at low frequencies, since genetic functions on the R factor essential for transfer are repressed; mutant "de-repressed" R factors which can transfer at high frequency are well known (Egawa and Hirota, 1962). However, most transfer in a repressed culture is not due to the appearance of such mutants, but to a small proportion of the population that becomes physiologically de-repressed (Watanabe, 1963; Meynell, Meynell and Datta, 1968). It is not known how long this state persists; it might, for instance, last for less than the cell cycle, and affect only a single cell, or it could persist through several cell cycles and thus be inherited clonally. The experiments reported here were designed to detect any such clonal behaviour, but they suggest instead that physiological "de-repression" is essentially a transient phenomenon, characterised by the transfer of a number of R factors to different recipient cells from a single donor.

The donor strain was the MetB$^-$ T6^S *Escherichia coli* strain ED1106, a derivative of the K12 strain W1655 (Lederberg and Lederberg, 1953) carrying the R100 plasmid (Egawa and Hirota, 1962), which confers tetracycline resistance. The recipient was *E. coli* strain ED1108, a prototrophic T1^R derivative of the T6^R streptomycin resistant K12 strain JC3051 (Achtman *et al.*, 1971).

An overnight culture of the donor in L broth was diluted 6×10^6-fold into 100 ml L broth in a 250 ml Erlenmeyer flask. The flask was vortexed vigorously and samples (0.3 ml) containing about 50 cells each were dispensed into 50 small tubes (set A); these and the remaining undispensed culture were incubated at 37° for 290 minutes, which allowed about 10 generations growth. The bulk culture was then vortexed again, and, while incubation continued, more samples (0.3 ml) were dispensed into a second set of 50 tubes (set B), which were also incubated at 37°. At 305 minutes, both sets were placed on ice while samples (0.1 ml) from each tube were diluted into buffer (10 ml) in preparation for viable count measurements. This and subsequent operations were performed in parallel for sets A and B. The cultures were replaced at 37°, and after a further 5 minutes incubation, the recipient cells were added to each tube (0.2 ml L broth culture containing 2×10^8 cells/ml.). Mating was allowed to proceed for 60 minutes; during this period, 0.1 ml samples from the dilutions made earlier were spread on nutrient

Table 1. Properties of the distributions of the donors and progeny, and the calculated number of progeny per active donor

Experiment	Set A					Set B				
	Distribution of viable counts of ED1106 per tube		Distribution of progeny per tube		Apparent progeny of active donor event	Distribution of viable counts of ED1106 per tube		Distribution of progeny per tube		Apparent progeny of active donor event
	Mean ($\times 10^{-4}$)	Variance ($\times 10^{-7}$)	Mean	Variance		Mean ($\times 10^{-4}$)	Variance ($\times 10^{-7}$)	Mean	Variance	
1	5.5	18.8	42.1	400	9.5	8.8	12.9	43.3	432	9.9
2	2.8	5.3	33.5	409	12.2	3.9	3.7	22.2	199	9.0
3	4.7	5.2	14.6	93	6.4	3.2	1.6	9.7	52	5.4
4	3.4	5.9	80.7	906	11.2	4.7	6.8	85.0	694	8.2

plates to obtain the viable counts of the donor populations in each tube. Mating was terminated by adding a suspension (0.2 ml) of phage T6 (which had been irradiated by ultraviolet light at a dose of 2500 ergs/cm^2) to give a final concentration of 1.3×10^9 phage/ml. After twenty minutes, by which time the phage had killed 99% of the donor cells, the cultures were streptomycin plated on minimal agar plates supplemented with tetracycline (10 μg/ml) and strepzomycin (2 mg/ml) to select for the progeny ED1108 cells now carrying the R100 factor. Control experiments showed that even in the absence of phage, the formation of new mating pairs on the plates could not account for more than 0.2% of the tetracycline-resistant streptomycin-resistant progeny obtained.

The progeny yields and their distributions from sets A and B in each experiment are similar (Table 1). If the ability to transfer the R100 factor was mainly a long-lived property, the occurrence of an active donor early in any tube of set A could give rise to a clone of active donors (up to about 1000 cells) and a high yield of progeny in that tube. This situation cannot occur in the tubes of set B since they contain samples dispensed from the bulk culture shortly before mating is started. The range of the viable counts from each B set serves as a measure of the variation introduced into all samples by the experimental manipulations; the relative standard errors range from 12–17%. The observed distribution of progeny among the tubes of each set would be expected to have about the same relative errors due to similar experimental manipulations, and therefore any large fluctuations in the number of progeny should have been detected easily. The largest number of progeny observed corresponded to 204 progeny in a single tube in set A of experiment 4; the average number of progeny per tube in this set was 80.7. The only other relatively large yields were those shown in Fig. 1 A and B. These values possibly represent the tail of a continuous distribution, and statistical analysis would be needed to assess their significance.

Since the progeny yields in the A sets show a similar variability to those in the B sets the active donor state does not show significant clonal multiplication, and is presumably somewhat transient. This conclusion contrasts, of course, with those drawn from experiments analysing the occurrence of more stable events

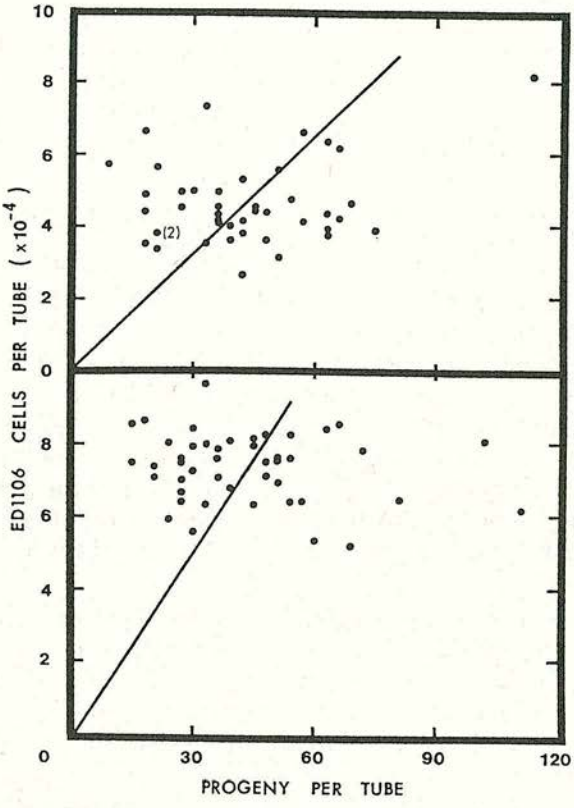

Fig. 1. Plots of viable counts of donor strain ED1106 per tube against progeny ED1108 cells carrying R100 per tube for set A (top) and set B (bottom) from Experiment 1 (Table 1)

such as mutation (Luria and Delbrück, 1943) and Hfr formation (Jacob and Wollman, 1956) where the populations of cells separated earlier show the greater variance in the distribution of mutants or Hfr cells observed, respectively.

The donor state, arising at random in the strain ED1106 population, should lead to approximate proportionality between numbers of progeny and the numbers of donors per tube. However, this was not observed. Figure 1 shows plots of progeny against viable count, together with the theoretical lines for perfect proportionality. The twofold variation in viable counts should be reflected in a twofold range in the numbers of progeny; the range observed is much larger. Although the numbers of progeny are small, they are estimated from counts obtained from one third of the total mating mixture, and can be considered reliable. The enhanced range must have some other origin than sampling errors. The lack of simple dependence of the progeny counts on the viable counts of donor cells and the wide range of progeny counts obtained both suggest that the progeny do not arise individually as the result of some random event activating a donor cell. Instead, we deduce that the appearance of progeny cells must be

inter-related events—in other words, the fundamental event that occurs within the culture produces a number of progeny cells. Since it is simplest to imagine the activation step as one that initially involves only a single cell, then one activated cell produces (directly or indirectly) a number of progeny rather than a single one; this number depends on the duration of the active state in the donor during the mating, on the availability of recipients and on the time at which the donor state appears in the mating mixture. We can estimate this number of successful transfers in the following manner. If the events leading to the active donor state occur at random among the tubes, they will have a Poisson distribution, and the mean and variance will be equal (see Feller, 1950). The ratio of the measured variance to the mean gives the number of progeny formed per activation event. Using this analysis, for example, Edwards and Meynell (1969) found that in chromosome marker transfer mediated by de-repressed mutants of colicin factor I this ratio approximated to unity, as though each recombinant arose independently. However, in our case this ratio ranges from 5–12, whether the tubes were inoculated with donor cells early (set A) or late (set B) (Table 1). If the large values noted above are removed from the distributions, a better fit to a Poisson distribution can be achieved. These latter values may therefore be evidence of a more fertile donor state.

There may be several contributory causes for the appearance of multiple progeny from a single transient activation event. Clonal inheritance of a short-lived activated state has already been concluded to be of minor significance. The number of progeny could increase either by growth and division of the cells, or by the primary progeny acting as new donors and re-transferring the R factors to other recipient cells. In a control experiment, in which phage T6 had been added to kill cells of strain ED1106 20′ after the start of the mating, little increase in the number of progeny was detected in the following 2 hours. Thus recently infected cells can neither retransfer or grow to any great extent, and under these experimental conditions, neither growth nor retransfer can therefore explain why multiple progeny arise from each de-repressed donor cell.

We conclude that the average active donor must have given rise directly to a number of exconjugant progeny. Two possibilities can be envisaged: the recipient cells can receive R factors from a single donor either simultaneously or sequentially (or through a combination of these modes); the present experimental evidence cannot distinguish between these alternatives. In either event, it follows that the number of active donors in the population is less than would appear to be the case if each donor gave only one progeny cell. The physiological origin of multiple transfers from a single donor cell could lie in the multinucleate state of the donor cells grown in broth; an R factor is probably associated with each nucleus, and the entire set could become available for transfer if the cell became physiologically "de-repressed".

It is evident that for detailed interpretation of fluctuation tests involving short-lived states analysis of the relation between donor counts and progeny yields can give valuable information that has not been available before.

Acknowledgement. I am very grateful to D. J. Finnegan and J. F. Collins for their help.

References

Achtman, N., Willetts, N., Clark, A. J.: Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterisation of transfer-deficient mutants. J. Bact. **106**, 529–538 (1971)

Edwards, S., Meynell, G. G.: I sex factors and chromosomal recombination in *Salmonella typhimurium*. Genet. Res. Camb. **13**, 321–323 (1969)

Egawa, R., Hirota, Y.: Inhibition of fertility by multiple drug-resistance factors in *Escherichia coli* K12. Jap. J. Genet. **37**, 66–69 (1962)

Feller, W.: An introduction to probability theory and its applications. Vol. I. New York: Wiley and Sons 1950

Jacob, F., Wollman, E. L.: Recombinaison génétique et mutants de fertilité chez *E. coli* K12. C.R. Acad. Sci. (Paris) **242**, 303–306 (1956)

Lederberg, E., Lederberg, J.: Genetic studies of lysogenicity in *E. coli*. Genetics **38**, 51–64 (1953)

Luria, S. E., Delbrück, M.: Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**, 491–511 (1943)

Meynell, E., Meynell, G. G., Datta, N.: Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bact. Rev. **32**, 55–83 (1968)

Watanabe, T.: Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. VI. High frequency resistance transfer system in *Escherichia coli*. J. Bact. **85**, 788–794 (1963)

Communicated by W. Maas

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PLASMID 1, 536–544 (1978)

Factors Affecting the Kinetics of Progeny Formation with F′*lac* in *Escherichia coli* K12

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Accepted June 12, 1978

In matings of F′*lac* donors with an excess of recipient cells, different donor cells mated at different times. The concentration dependence of mating is incompatible with bimolecular reaction kinetics. In exponentially growing cultures, F′*lac* transfer from each donor cell continues to occur about once per generation. The establishment of F′*lac* in some recipient cells may take more than five generations.

The analysis of the "epidemic spread" of plasmids through bacterial populations depends upon distinguishing (i) primary transfer from donor cells and (ii) the ability of recipients to become donors themselves. The principal factors affecting the process are given in Table 1. By following the kinetics of mating and by plating suitable dilutions of samples on selective agar after using different regimes, we have assessed the relative importance of the factors given in Table 1. In this paper we are concerned only with mating by the original donor strain. The results here are then used in conjunction with those from more long-term matings, for the analysis in the following paper (Cullum *et al.*, 1978) of further spread of the plasmid in the recipient population.

It is likely that collision and pair formation in F′ and Hfr conjugation are very similar as they both involve the expression of the F transfer genes in the donor cell. Collins and Broda (1975) studied the effects of cell concentration on motility and mating pair formation (measured by progeny yield). They discovered that motility, and hence collision rate, was lower at a concentration of 4×10^8 cells/ml than at 3×10^6 cells/ml. The efficiency of pair formation after collision was also much lower at the higher concentration. A reduction in aggregate-forming ability at high concentration was also observed by Eckerson and Reynard (1977) using R1-19. Because of the possibility of deviations from an ideal bimolecular reaction, we studied the effects of concentration on progeny yield in F′*lac* matings.

Broda (1975) showed that each transiently derepressed donor of the repressed F-like plasmid R100 gives rise to about 10 progeny in 60-min matings. We used a similar method to find out whether some F′*lac* donors can transfer more than once in a 30-min period. We also looked at the related question of whether there was a period of impotence after mating during which a donor cannot mate again.

It is possible for a recipient to mate with more than one donor during a short time, as is shown by progeny cells that receive DNA from two different donor cells in mixed matings (Scaife and Gross, 1962; Fischer-Fantuzzi and di Girolamo, 1961). If this occurred frequently, it would complicate the analysis as only one progeny would be formed as the result of several donors' mating. We avoided the problem by performing matings with an excess of recipients. This also ensured that the shortage of recipients competent to mate was never a limiting factor.

Events in the recipient may be different in F′ and Hfr matings, because some F genes cannot be transferred to the recipient until very late in Hfr matings (Broda *et al.*, 1972)

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TABLE 1

FACTORS AFFECTING PROGENY NUMBER IN PLASMID MATINGS

A. Factors affecting transfer
   (1) Collision rate of donor and recipient cells
   (2) Donor mating ability
   (3) Recipient mating ability

B. Factors acting after transfer
   (1) Segregation of plasmidless cells on division of recipients that have received the plasmid
   (2) Effects of carrying the plasmid on recipient growth
   (3) Retransfer of the plasmid by recipients

and because of the different types of recombination events that occur after Hfr and F′ transfer. De Haan and Stouthamer (1963) showed that new recipients of an F′*gal* could produce sectored Gal^+/Gal^- colonies, which suggested that F^- cells were segregated by such new progeny. If such segregation occurred often, the apparent growth rate of new progeny would be reduced. It would be difficult to separate this effect from any real short-lived effect on the growth rate. We therefore measured the apparent growth rate of new progeny and studied the composition of a few sectored colonies.

Achtman *et al.* (1971) found that retransfer of F′*lac* by recipients was insignificant in matings of less than 50-min duration at 42°C. We measured the time needed for retransfer to become important in our system at 37°C.

MATERIALS AND METHODS

Media. Bacteria were grown and mated in L-broth which contains (per liter): 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl. It was adjusted to pH 7.2 with NaOH. Care was taken to keep the L-broth detergent free, because of the sensitivity of donors to detergent (Tomoeda *et al.*, 1975). All dilutions were done in L-broth.

The nutrient agar used was L-broth solidified with 1.5% agar (Difco). Lactose tetrazolium agar was made by adding 1% lactose and 0.002% tetrazolium dye to L-broth agar.

For minimal agar, Spizizen salts (X5) [per liter: $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 14 g; KH_2PO_4, 6 g; trisodium citrate, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g] was added 1:4 to 2% agar (Davis, N.Z.) together with the appropriate supplements. Thiamine, 1 μg/ml, and an additional 1.25 mM $MgSO_4$ were also added. The other supplements were added at the following concentrations: sugars (glucose and lactose), 2 mg/ml; amino acids (except lysine), 20 μg/ml; lysine, 100 μg/ml; streptomycin sulfate 200 μg/ml; nalidixic acid (Sigma), 40 μg/ml.

Top agar was 0.7% Difco agar and was dispensed into tubes in 3-ml amounts.

Bacterial strains and growth conditions. The bacterial strains are shown in Table 2. All incubations were at 37°C, with shaking of liquid cultures, except where mentioned. We inoculated about 1 in 100 from standing overnight cultures into sidearm flasks of 10 times the culture volume. These cultures were incubated until they had reached slightly less than half the required cell concentration and were then stored on ice. Then the cultures were reincubated after the ice treatment for about 40 min before use.

Matings to find effect of parental concentrations. One-milliliter volumes of ap-

TABLE 2

BACTERIAL STRAINS[a]

Strain	Properties					Sex
	Str	Nal	T6	His	Su	
JC6582	S	S	S	+	+	F′*lac*$^+$*tra*$^+$
M172	S	S	S	+	+	F′*lac*$^+$*traH88*
JC3272	R	S	R	−	−	F^-
ED2196	S	R	R	−	−	F^-

[a] The strains were kindly provided by Dr. N. Willetts (see Achtman *et al.*, 1971). They all have a requirement for tryptophan and all carry the *lac* deletion X74. The phenotype abbreviations are as follows: Str, streptomycin resistance; Nal, nalidixic acid resistance; T6, resistance to phage T6; His, nonrequirement of histidine; Su, capacity to suppress *traH88*.

propriate dilutions of exponentially growing cultures of JC6582 and ED2196 were mixed in 18 × 150-mm tubes and incubated for the required mating time (20 or 30 min). Samples of 0.1 ml of appropriate dilutions were plated, using top agar, on selective agar. Nalidixic acid was included as a counter selective agent that prevented F*lac* transfer occurring on the plates (Barbour, 1967).

Matings to find multiplicity of mating. A 0.1-ml volume of the required JC6582 dilution was put into each of 50 12 × 75-mm tubes which were incubated without shaking. After 5 min of incubation, 0.1 ml of an exponentially growing ED2196 culture was added to each tube. After 30 min of mating, 0.1 ml of phage T6 (about 10^9 PFU) was added to each tube. The rapid dispensing of the cultures and phage was achieved by using a Colworth Droplette. After 5 min, the total contents of each tube were plated, using top agar, on agar selective for Lac^+Nal^R progeny. Plating all the tube cultures took 7 min.

Preparation of high-titer T6. Lysates of T6 grown on ED2520 (an *Escherichia coli* B/r/1 strain) were centrifuged for 60 min at 4°C at 19,000 rpm in a Beckman Type 19 rotor. The supernatant was discarded and the pellet was resuspended in phage buffer [per liter: Na_2HPO_4, 7 g; KH_2PO_4, 3 g; NaCl, 5 g; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; gelatin, 0.001%].

Lac^+/Lac^- sectored colonies. We mixed equal volumes of exponentially growing cultures of the donor and recipient strains at a concentration of about 1.5×10^8 cells/ml. After 20 and 30 min of mating, samples were diluted in L-broth, blended, and spread on fresh lactose tetrazolium agar and fresh L-broth agar (both containing streptomycin). The experiment was performed in a room at 37°C, with media and equipment prewarmed to minimize disturbance of the cells.

After overnight incubation, the lactose tetrazolium plates were examined for the presence of sectored Lac^+/Lac^- colonies (i.e., white/red sectors). Dilutions were chosen to give 50–100 colonies per plate.

Colonies on the L-broth agar were tested for β-galactosidase production as follows. Whole well-isolated colonies were put into tubes containing 2 ml of L-broth, which were Vortex-mixed to emulsify the colonies; the tubes were stored at 4°C while the test for β-galactosidase activity was performed. Samples of 0.1 ml were added to tubes containing 1 ml of minimal medium supplemented with casamino acids, tryptophan, and 1% lactose. These tubes were incubated at 37°C for 2 h. The production of β-galactosidase was then tested using a procedure adapted from Pardee *et al.* (1959). One drop of toluene was added to each tube and the tubes were shaken vigorously at 37°C for 30 min to evaporate the toluene. Then 0.3 ml of M/75 *o*-nitrophenyl-β-D-galactoside (BDH) was added to each tube and incubation at 37°C was continued. Most colonies containing Lac^+ cells gave a yellow color within 15 min. Incubation was continued overnight to detect smaller proportions of Lac^+ cells. Tests showed that this procedure would detect at least as few as 1% Lac^+ cells.

Appropriate dilutions of the emulsified colonies which produced β-galactosidase were plated on lactose tetrazolium agar to estimate the proportions of Lac^+ and Lac^- cells.

RESULTS

Effect of Donor and Recipient Concentrations on Progeny Formation

We mated JC6582 for 30 min with ED2196 in tubes and measured the number of F*lac* progeny formed per donor. The recipient (ED2196) concentration was kept constant (at 2×10^8 cells/ml) and the donor concentration was varied from 2×10^4 to 2×10^8 cells/ml. Over most of the range about one progeny colony arose per donor cell. In matings where the donor and recipient concentrations were similar (donor concentration above 5×10^7/ml) the number of progeny per donor was reduced to about 0.3. We therefore performed most of our matings with at least a 10-fold excess of recipients.

Figure 1 shows the results of mating (for both 20 and 30 min) a low concentration of donors (about 10^4 cells/ml) with recipients at concentrations ranging from about 10^5 to 2×10^8 cells/ml. In the 30-min matings, the number of progeny per donor reaches a plateau of about 1 when the recipient concentration is increased to above 2×10^7 cells/ml, presumably because collision then ceases to be the limiting factor. If the kinetics of mating were analogous to the kinetics of a bimolecular reaction, then at lower recipient concentrations the number of progeny per donor should be proportional to the recipient concentration. However, Fig. 1 shows that, as recipient concentration is reduced 200-fold from 2×10^7 to 10^5 cells/ml, the number of progeny per donor falls only 30-fold. Thus, the kinetics of mating appear to be different from the kinetics of bimolecular reactions.

The 20-min matings also give a plateau, but this plateau is about 0.4 progeny per donor. Since the collision rate is not the limiting factor at these concentrations, this lower value indicates that there are many donors that can mate in 30 min but not in 20 min.

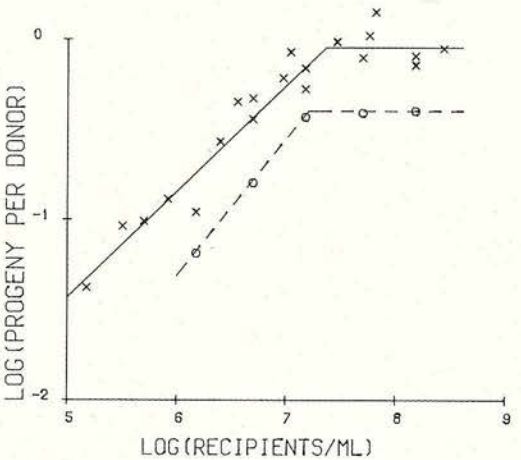

FIG. 1. Effect of recipient concentration on F'*lac* matings. JC6582 was mated with ED2196 in tubes for 20 and 30 min. The recipient concentration at the start of the mating was varied between about 2×10^5 and 2×10^8 cells/ml. The donor concentration was about 2×10^4 cells/ml. Selection was for $Nal^R Lac^+$ colonies. (○) Number of progeny per donor after 20-min mating; (×) number of progeny per donor after 30-min mating.

Multiplicity of Donation

We showed above that matings under suitable conditions gave rise to a number of progeny similar to the number of donors after 30 min. This could be due to most donors in the population mating with one recipient or to a small number of very fertile donors.

We tried to distinguish these possibilities by using an experiment similar to that of Broda (1975). We added 0.1 ml of a dilution of a donor (JC6582) culture to a series of small tubes; the dilution was chosen to give an average of about one donor cell per tube. After 5 min of incubation, 0.1 ml of a recipient (ED2196) culture was added to each tube. After 30 min of mating, a high titer of phage T6 was added to each tube to kill the donor and stop mating. After 5 min of further incubation, the total contents of each tube were plated on agar selective for the Lac^+Nal^R progeny. We used matings of the donor culture at 100-fold the concentration as a control to measure the efficiency of mating and to measure donor growth.

If the fertility of donor cells does not vary much from cell to cell, the variance of the number of progeny obtained from each tube should be relatively small; if, on the other hand, a few very fertile donors were responsible for much of the transfer, the variance would be much higher. Table 3 shows that the number of progeny per donor in the control matings is about 1 and the average number of progeny in the series of tubes is about one-hundredth of that in the control, so that the efficiency of mating is the same. The donor numbers in the controls increase about fourfold during mating.

If the number of donors entering each tube followed a Poisson distribution (i.e., mean and variance equal), and if each donor entering a tube produced four progeny, the variance of the number of progeny in each

TABLE 3

VARIATIONS IN DONOR FERTILITY IN DUPLICATE JC6582 × ED2196 MATINGS

	Experiment A	Experiment B
Control matings (donor concentration × 100)		
Average number of donors entering tubes	92.3	87.8
Average number of donors after mating	470	396
Average number of progeny per tube	398	456
Number of progeny per donor after mating	0.85	1.15
Recipient concentration ($\times 10^{-8}$/ml)	1.5	2.1
Tubes with low donor numbers		
Average number of progeny per tube ($\bar{x}$)	3.88	4.47
Variance of number of progeny (s^2)	10.1	18.8
$s^2/\bar{x}$	2.61	4.47
Number of tubes	50	49

tube would be four times the mean. Thus, the results of the two experiments shown in Table 3 show a variance of about the amount expected if there were no differences in fertility between donors. As the mean number of progeny per tube is close to that expected from the controls, any rare events due to more fertile donors do not contribute much to the observed number of progeny.

Time between Rounds of Transfer

The facts that most donors seem to transfer to only one recipient in 30 min (see above), and that a plateau of progeny per donor is reached with increasing recipient concentration (Fig. 1) suggest that there is a time lag between rounds of F'*lac* transfer.

We did an experiment (Fig. 2) to establish directly whether such a lag exists. JC6582 was mated with equal numbers of two recipients in the same mating mixture. In both cases, JC3272 was present throughout the mating. In one case, ED2196 was present at the start of mating, while in the other it was not added until 20 min later to allow most of the donors to pair. It took about 50 min after the addition of ED2196, in the second case, for the number of Lac^+Nal^R progeny to reach a level comparable to that of the donor, JC6582. This compares with the 20 min needed when the donor had not previously mated (i.e., for the ED2196 in the first case and for JC3272 in both cases). This suggests that there is a lag of about 30 min between rounds of transfer. However, the number of progeny of the second recipient has already reached 10% of the donor numbers within 30 min of adding the second recipient. This early transfer must be from the original donor strain as the experiment described in the next section shows that retransfer from the first recipient cannot occur so soon.

Retransfer by Recipients

Transfer can occur both from the original donor and from recipients that have received

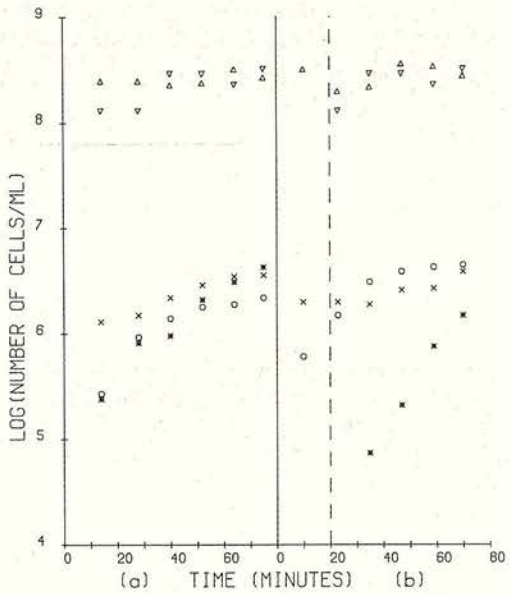

FIG. 2. Time between rounds of F'*lac* transfer by donors. JC6582 was mated with a mixture of approximately equal numbers of the two recipient strains, JC3272 and ED2196. (a) Both recipients were present at the start of mating; (b) ED2196 only was added after 20 min of mating. (×) JC6582 donor, (△) JC3272 recipient, (▽) ED2196 recipient, (○) JC3272 (F'*lac*) progeny, (*) ED2196 (F'*lac*) progeny.

the plasmid earlier. Such "secondary" transfer is not important in matings lasting less than 90 min (Fig. 3). We mated JC6582, which is $T6^S$, with JC3272, which is $T6^R$. After 20 min, a high titer of phage T6 was added. After a further 10 min, a sample of the mating mixture was added to a culture of a second $T6^R$ recipient, ED2196, and transfer of F*lac* from JC3272 was followed. Although low levels of transfer can be detected within 40 min, most cells are only able to mate after about 90 min.

Growth and Segregation in New Progeny

Any disturbance in the growth and division of new progeny or any segregation of F^- cells will affect the apparent progeny growth rate and therefore complicate our analysis. We tried to measure directly the apparent growth of new progeny in matings between JC6582 and JC3272 in which the donor was killed after 17 min with phage T6. Figure 4 shows that the apparent growth rate of the progeny was slightly lower than that of recipient cells in the first 20 min after the T6 was added. However, the sampling errors inevitably associated with this type of experiment make it difficult to interpret effects of this magnitude.

We also did an experiment to look for Lac^+/Lac^- sectored colonies as evidence for F^- segregants from JC3272 cells that had received F*lac* (as in de Haan and Stouthamer, 1963). JC6582 was mated with JC3272 for 20 min and blended samples were plated on lactose tetrazolium agar containing streptomycin. About 10% of the white (Lac^+) colonies had red (Lac^-) sectors; no Lac^+ sectors were seen in predominantly red colonies. Since we were concerned that intracolony mating on the agar could reduce the number of sectored colonies, we repeated the experiment using M172 as the donor. M172 carries an F*lac* that carries an amber mutation in the transfer gene *traH*, so that the progeny were transfer deficient. This experiment showed that intracolony mating was unimportant. This is consistent with the work of Broda and Collins (1978) on Hfr matings.

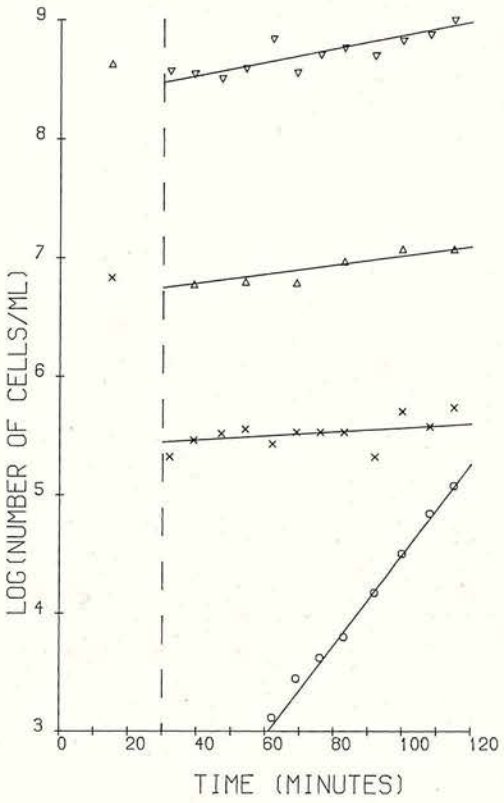

FIG. 3. Time needed for retransfer of F'*lac* by recipients. JC6582 was mated with JC3272 for 20 min. The primary donor, JC6582, was killed by the addition of sufficient phage T6 to give a final concentration of 10^{10} PFU/ml. At 30 min, a sample from the mating mixture was added to an ED2196 culture to measure retransfer of the plasmid by the JC3272 (F'*lac*) progeny. (△) JC3272 recipient, (▽) ED2196 recipient, (×) JC3272 (F'*lac*) progeny, (○) ED2196 (F'*lac*) progeny.

Although sectored colonies that contained a high proportion of both Lac^+ and Lac^- cells should be easily detected, it was possible that those sectored colonies containing only a few Lac^- cells would not have been detected by inspection only, so that the proportion of the recipient population that was mixed with respect to the Lac phenotype would be underestimated. We also wanted to use the distribution of ratios of Lac^+ to Lac^- cells in mixed colonies to find out when segregation occurred; this was difficult using

lactose tetrazolium agar because Lac$^+$ cells grow faster. We therefore performed a mating in which we also plated samples on L-broth agar containing streptomycin and (as suggested by Dr. J. Scaife) tested 60 colonies for β-galactosidase production. We then measured the ratio of Lac$^+$ to Lac$^-$ cells in colonies found to produce the enzyme (Table 4).

In those colonies that had more than 98% Lac$^+$ cells any segregation of Lac$^-$ cells would have had to occur more than six generations after plating. Such segregation would have no significant effect on the progeny growth rate if it occurred in liquid culture. Some of the mixed colonies with such low numbers of Lac$^-$ cells would probably not appear as sectored on lactose tetrazolium agar.

Four out of eleven progeny colonies from the 20-min mating had a significant proportion (more than 25%) of Lac$^-$ cells. If this corresponds to what happens in liquid cultures, then the apparent growth rate of new progeny will be reduced by about a third compared to the recipient growth rate. The number of colonies tested was not large enough to show whether there was a difference between the 20- and 30-min samples.

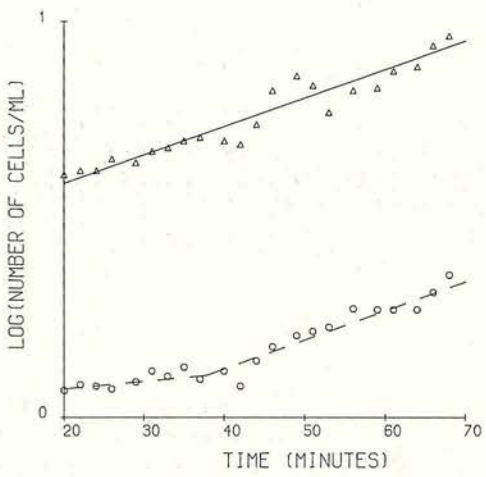

FIG. 4. Growth of new progeny. JC6582 was mated with JC3272 for 17 min, and then phage T6 was added to kill the donors. The growth of the recipient and progeny was then followed. The data were smoothed by taking the geometric mean of the value at each time point with those at the neighboring two time points. (○) JC3272 (F'*lac*) progeny ($\times 10^{-6}$); (△) JC3272 recipient ($\times 10^{-8}$).

TABLE 4

MIXED Lac$^+$/Lac$^-$ COLONIES IN MATINGS BETWEEN JC6582 AND JC3272[a]

	20-min mating	30-min mating	Total
(1) β-Galactosidase test			
Number of colonies tested	40	20	60
Number producing β-galactosidase	11	7	18
(2) Proportion of Lac$^+$ cells in colonies producing enzyme			
More than 98%	7	6	13
75–98%	0	0	0
25–75%	2	0	2
2–25%	0	0	0
Less than 2%	2	1	3
(3) Colonies on lactose tetrazolium agar			
Number of colonies examined	204	215	419
Number of Lac$^+$ colonies (including sectored colonies)	29	40	69
Number of sectored colonies	3	2	5

[a] Note: If it is assumed that the colonies with less than 2% Lac$^+$ cells would appear Lac$^-$ on the tetrazolium agar, the proportion of Lac$^+$ colonies by β-galactosidase assay (15/60) is not significantly different from that (69/419) using lactose tetrazolium agar ($\chi_1^2 = 2.6$; this is not significant at the 10% level).

DISCUSSION

Walmsley (1973) calculated that only 30% of recipients in his Hfr matings were competent to form mating pairs; however, his assumption that pair formation is analogous to a simple bimolecular process is not supported by the measurements of Collins and Broda (1975). In most of our matings the recipient cells were in excess, so that in any event problems of recipient mating competence were avoided. We found that the

recipient concentration is not a limiting factor in mating when it is above 2×10^7 cells/ml (Fig. 1). This suggests that, at these concentrations, the collision rate is high enough for donor fertility to become the controlling factor. Mating appears to be more efficient at low cell concentrations, as was previously reported for Hfr matings by Collins and Broda (1975) who attributed it in part to lower motilities at high cell concentrations.

The results in Table 3 show that there are not great differences in fertility between individual donor cells that are growing exponentially. This does not rule out a donor's mating several times with the same recipient, but it does rule out plasmid transfer to many recipients and then low probability of inheritance; such behavior would give a higher variance.

This monogamy and the time lag between rounds of transfer by donors might be due to donors being unable to mate with a second recipient until the mating complex with the first recipient has disaggregated. Such disaggregation should occur within 60 min of the mixing of the donor and recipient culture (Achtman, 1977). Alternatively, donors might only donate during a certain, fairly short, period of the cell cycle. The lag between rounds of transfer would then be one generation time. This would also explain the fact that some donors that are unable to mate in 20 min can mate in 30 min (Fig. 1) and the spread in times between mating aggregate formation and F'*gal* transfer observed by de Haan and Stouthamer (1963).

Since 90 min elapse before most new progeny become efficient donors (Fig. 3) retransfer by recipients is relatively unimportant in the analysis of matings of short duration. Comparable results were reported by Achtman *et al.* (1971). The delay presumably reflects the need to synthesize sufficient quantities of transfer products; relatively large quantities of sex pilus protein must be made and this is a complicated process involving several modification steps (for a review, see Tomoeda *et al.* 1975).

Progeny cell numbers soon increase at the rate characteristic of the recipient strain (Fig. 4), even though new progeny growing on agar can segregate F^- cells. The colonies (Table 4) that contained less than 2% of Lac^+ cells would correspond to the unilinear inheritance of F'*lac* for six or more generations. De Haan and Stouthamer (1963) found sectored colonies even in samples plated 60 min after mating had been interrupted, so that similar behavior probably occurs in liquid culture. We do not know whether this unilinear inheritance is due to properties of the plasmid DNA transferred or to the state of the recipient.

Finally, the results in Table 4 seemed to suggest that there might be a difference in lac^+ progeny counts according to whether one scored on lactose tetrazolium agar or by β-galactosidase production. While the difference observed was not statistically significant, the fact remains that, in this as in all other such genetic transfer experiments, the conclusions reached are heavily dependent on the reliability of the technique used for scoring the transfer events. As the reliability of scoring *lac* transfer on lactose tetrazolium plates has been verified many times, we are confident that the results in Table 4 reflect statistical fluctuations and not unreliability of the method.

ACKNOWLEDGMENTS

We thank the Medical Research Council for support in the form of a training grant (to J.C.) and a project grant (to J.F.C. and P.B.). We thank Helen Grozier for technical assistance.

REFERENCES

ACHTMAN, M. (1975). Mating aggregates in *Escherichia coli* conjugation. *J. Bacteriol.* **123,** 505–515.

ACHTMAN, M. (1977). A physical analysis of mating in *Escherichia coli*. *In* "Plasmids: Medical and Theoretical Aspects" (S. Mitsuhashi, L. Rosival, and V. Krimery, eds.), pp. 117–125. Avicenum, Prague/Springer, Heidelberg.

ACHTMAN, M., WILLETTS, N., AND CLARK, A. J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterisation of transfer-deficient mutants. *J. Bacteriol.* **106,** 529–538.

BARBOUR, S. D. (1967). Effect of nalidixic acid on conjugational transfer and expression of episomal Lac genes in *Escherichia coli* K12. *J. Mol. Biol.* **28,** 373–376.

BRODA, P. (1975). Transience of the donor state in an *Escherichia coli* K12 carrying a repressed R factor. *Mol. Gen. Genet.* **138,** 65–69.

BRODA, P., AND COLLINS, J. F. (1978). Role of simple and complex aggregates in *Escherichia coli* Hfr × F⁻ matings. *Genet. Res., Cambridge* **31,** 167–175.

BRODA, P., MEACOCK, P., AND ACHTMAN, M. (1972). Early transfer of genes determining transfer functions by some Hfr strains in *Escherichia coli* K12. *Mol. Gen. Genet.* **116,** 336–347.

COLLINS, J. F., AND BRODA, P. (1975). Motility, diffusion and cell concentration affect pair formation in *Escherichia coli. Nature (London)* **258,** 722–723.

CULLUM, J., COLLINS, J. F., AND BRODA, P. (1978). The spread of plasmids in model populations of *Escherichia coli* K12. *Plasmid* **1,** 000–000.

CURTISS, III, R., CARO, L. G., ALLISON, D. P., AND STALLIONS, D. R. (1969). Early stages of conjugation in *Escherichia coli. J. Bacteriol.* **100,** 1091–1104.

DE HAAN, P. G., AND STOUTHAMER, A. H. (1963). F-prime transfer and multiplication of sexduced cells. *Genet. Res. Cambridge* **4,** 30–41.

ECKERSON, H. W., AND REYNARD, A. M. (1977). Effect of entry exclusion on mating aggregates and transconjugants. *J. Bacteriol.* **129,** 131–137.

FISCHER-FANTUZZI, L., AND DI GIROLAMO, M. (1961). Triparental matings in *Escherichia coli. Genetics* **46,** 1305–1315.

LOW, B., AND WOOD, T. H. (1965). A quick and efficient method for interruption of bacterial conjugation. *Genet. Res. Cambridge* **6,** 300–303.

PARDEE, A. B., JACOB, F., AND MONOD, J. (1959). The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *Escherichia coli. J. Mol. Biol.* **1,** 165–178.

SCAIFE, J., AND GROSS, J. D. (1962). Inhibition of multiplication of an F-*lac* factor in Hfr cells of *Escherichia coli* K12. *Biochem. Biophys. Res. Commun.* **7,** 403–407.

TOMOEDA, M., INUZUKA, M., AND DATE, T. (1975). Bacterial sex pili. *Progr. Biophys. Mol. Biol.* **30,** 23–56.

WALMSLEY, R. H. (1973). Physical assay of competence for specific mating-pair formation in *Escherichia coli. J. Bacteriol.* **114,** 144–151.

PLASMID 1, 545–556 (1978)

The Spread of Plasmids in Model Populations of *Escherichia coli* K12

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Accepted June 12, 1978

Comparison of R100 with its derepressed derivative R100-1 showed that the capacity to repress *tra* function does not significantly affect the spread by retransfer of R100. F'*lac* was used to investigate the contributions of growth and transfer to spread of a plasmid through a recipient population. Ability to transfer F'*lac* was lost rapidly when donor cultures entered stationary phase, but aggregate-forming ability was lost much more slowly. Comparison of F'*lac tra*$^+$ with F'*lac traH88*, which is unable to retransfer from recipients, showed the importance of retransfer. We used a mathematical model to calculate the amount of retransfer needed to explain the rate of increase of F'*lac* progeny. This showed that the lag between a cell receiving F'*lac* and being able to retransfer it was a less important constraint on this rate of increase than the inherent rate of plasmid transfer by established donors.

Most transmissible plasmids found in nature transfer very inefficiently. In some cases it is possible to isolate mutants that transfer much more efficiently showing that some kind of repression mechanism is involved (Meynell *et al.*, 1968).

Ozeki *et al.* (1962) found another method of obtaining highly fertile cultures using the repressed plasmid Col I in *Salmonella typhimurium*. They produced "high frequency of colicinogeny transferring" (HFCT) cultures by incubating particular ratios of colicinogenic and noncolicinogenic cells together for 20 h under conditions allowing slow growth. Such high fertility was due to the fact that recipient cells that had recently received the plasmid were efficient donors for several generations (Stocker *et al.*, 1963). Watanabe (1963) did similar experiments with an F-like R-factor 222 (also known as R100) in *Escherichia coli*. Here "epidemic spread" of the plasmid was apparently less efficient.

The proportion of a recipient population to which a plasmid spreads is determined by three factors.

(1) Initial transfer: In the early stages transfer by the initial plasmid-carrying donor cells will be the most important factor. It gives an initial number of progeny that may then retransfer the plasmid several times before repression sets in.

(2) Rate of spread of a plasmid by retransfer and growth: This rate can be expressed best as the rate of increase of progeny numbers divided by the number of progeny; this proportional rate of increase is given by the slope of the line of progeny numbers plotted against time on a semilogarithmic graph. This is because, under conditions of excess recipient numbers, we would expect both the number of new progeny produced by retransfer of the plasmid (see Cullum *et al.*, 1978) and the increase of progeny numbers due to growth to be proportional to progeny numbers. Transfer by the original donors would also continue; this would add a measure of uncertainty to the interpretations of the above slope.

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TABLE 1

BACTERIAL STRAINS

Strain	Properties[a]	Plasmid carried	Source
JC6582[b]	$T6^S$ Su_I^+	F'*lac tra*$^+$	N. Willetts (Achtman *et al.*, 1971)
M172[b]	$T6^S$ Su_I^+	F'*lac traH88*	N. Willetts (Achtman *et al.*, 1971)
JC3272[b,c]	His^- Lys^- $T6^R$ Str^R Su_I^-	None	N. Willetts (Achtman *et al.*, 1971)
ED2196[b]	His^- $T6^R$ Nal^R Su_I^-	None	N. Willetts
ED892[d]	Met^-	R100-1	This laboratory
ED1106[d]	Met^-	R100	Broda (1975)

[a] The phenotype abbreviations are as follows: $T6^R/T6^S$, resistance/sensitivity to phage T6; Su_I^+/Su_I^-, capable/incapable of suppressing *traH88*; His^-, Lys^-, Met^-, requirement for histidine, lysine, and methionine, respectively. Str^R, Nal^R, resistant to streptomycin and nalidixic acid, respectively.

[b] These strains all require tryptophan and have *lac* deletion ×74.

[c] JC3272 is also able to ferment maltose and has a defective λ prophage.

[d] These strains are W1655 derivatives. The plasmids all carry genes for tetracycline resistance.

(3) The time during which retransfer can occur: Eventually the conditions in a mating mixture become unsuitable for plasmid transfer.

In this paper we mainly consider the second factor. We first studied the effect of repression on the rate of spread of R100 and then attempted to separate the effects of transfer and growth using the derepressed plasmid F'*lac*.

MATERIALS AND METHODS

The bacterial strains are shown in Table 1.

The media, growth conditions, and the preparation of high-titer phage T6 stocks were described in Cullum *et al.* (1978). When required, tetracycline (Lederle) was added to the media to give a final concentration of 10 μg/ml. Liquid cultures were always grown in L-broth. Clones were tested for MS2 sensitivity by replica plating onto Giemsa agar spread with MS2 as described in Cullum and Broda (1978).

Coulter counter measurements. We used a Coulter counter model Z_B, with a 30-μm orifice, that was connected to a Coulter channelyser with a hundred channels to measure the aggregate-forming ability of a donor culture. An account of the operation of such an apparatus is given by Achtman (1975).

Samples of the donor cultures, diluted to the same optical density as the recipient were mated for 30 min in tubes with equal volumes of an exponential culture of ED2196 (about 1.5×10^8 cells/ml). A sample from the tube was gently diluted into L-broth containing 1.87 μm-diameter latex spheres (Coulter). Blended [with a mating interrupter (Low and Wood, 1965)] and unblended samples of the bacteria–latex mixture were examined in the Coulter counter.

For each sample, two peaks were seen with the channelyser. The peak at the larger particle size was due to the latex, and the other peak was due to bacteria. The concentration of latex was high enough for the contribution of cell aggregates to the latex peak of the unblended mixture to be negligible. The bacteria in the blended samples are present almost entirely as single cells and there was a cutoff size between the bacterial and latex peaks where the number of particles was approximately zero. The particles in the unblended mixture smaller than this cutoff size must have been mainly single cells, since there were very few particles of less than half the cutoff size in the sample, i.e., they were too small for pairs to have made a large contribution. The number of particles in the cell peaks of the blended (C+) and unblended (C−) mixtures and the

latex peaks of the blended (L+) and unblended (L−) mixtures were measured. The volumes of blended and unblended mixtures sampled by the Coulter counter were, in general, different because the mixtures were sampled until one of the channels of the Channelyser reached the maximum count of 10,000 particles. Control experiments showed that the latex is unaffected by blending; therefore the ratio of L+ to L− gives the ratio of volumes sampled in the blended and unblended samples. Then, the numbers of single cells (i.e., not in aggregates) in the two samples, C+ and C−, allows one to calculate the proportion of cells in aggregates as: $1 - (L+)(C-)/(L-)(C+)$.

The use of an internal latex control avoids sampling errors in dilution and in the volume sampled by the Coulter counter. The method is also insensitive to drift in machine settings and any bias by the Channelyser against larger particles.

The method used by Achtman (1975) was inappropriate for our experiment as the proportion of cells in the donor culture found in aggregates changed appreciably over the course of the experiment (from 7.5 to 17%) and the formula of Achtman (1975) gives only the number of parental "particles" involved in mating aggregates. Also, his assumption that every particle, regardless of size, was equally likely to participate in mating aggregates might not be appropriate for cultures entering stationary phase.

RESULTS

Effect of Repression

Preliminary experiments using low donor–recipient ratios in 20-h matings showed that our R100 donor strain (ED1106) could increase considerably in numbers (about a 100-fold) under conditions where the recipient (JC3272) viable count only doubled. We therefore had to study the kinetics of matings rather than just the initial and final numbers in the mating mixtures.

We performed parallel matings of strains carrying R100, and its derepressed derivative R100-1, with JC3272 to find out if the repression system affected the rate of spread of the plasmid. Initial donor numbers were chosen to give similar initial numbers of progeny carrying R100 and R100-1. Figure 1 shows that the rate of increase in progeny numbers for both R100 and R100-1 in the period 200 to 600 min is greater than the recipient growth rate. This is not due to differential growth rates because, although R100 increases the recipient growth rate, R100-1 decreases it (Willetts and Finnegan, 1970). Thus transfer must be occurring. This would be both transfer from the original donors and retransfer from the recipients. Of the two, retransfer from the recipients is probably the major component for two reasons.

(i) The ratio of progeny to donors increases more than five fold between 200 and 600

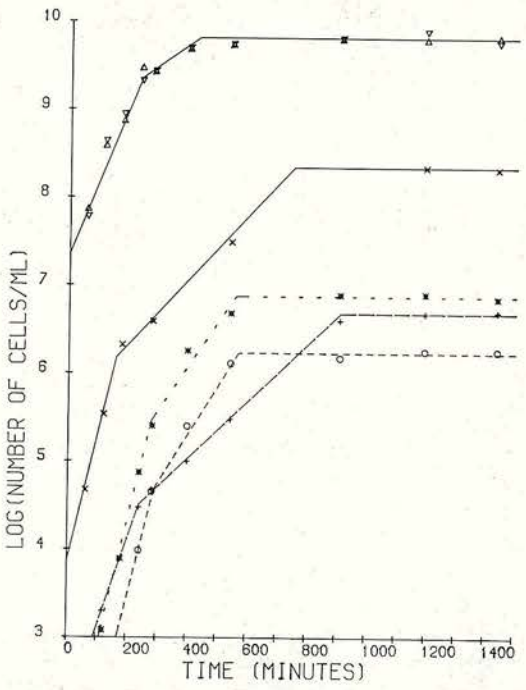

FIG. 1. Matings of ED1106 (which carries R100) and ED892 (which carries R100-1) with JC3272. Exponentially growing cultures of the parent strains were diluted in warm L-broth and mixed to start the matings. The progeny were selected as $Str^R Tet^R$ cells. ED1106 × JC3272: ED1106 donors (×), JC3272 recipients (△), JC3272 (R100) progeny (○). ED892 × JC3272: ED892 donors (+), JC3272 recipients (▽), JC3272 (R100-1) progeny (★).

min. If transfer in this interval were mainly from the primary donor strain, then its fertility would also have to increase by a comparable amount to sustain the increase in progeny numbers. This seemed implausible.

(ii) The progeny numbers stop increasing at about the same time as does the recipient population as a whole but long before the donor cells enter stationary phase. The recipient ability is probably not much affected by the growth conditions (see Burman, 1975). This suggests that transfer by the original donors, which would be expected to continue, is relatively unimportant.

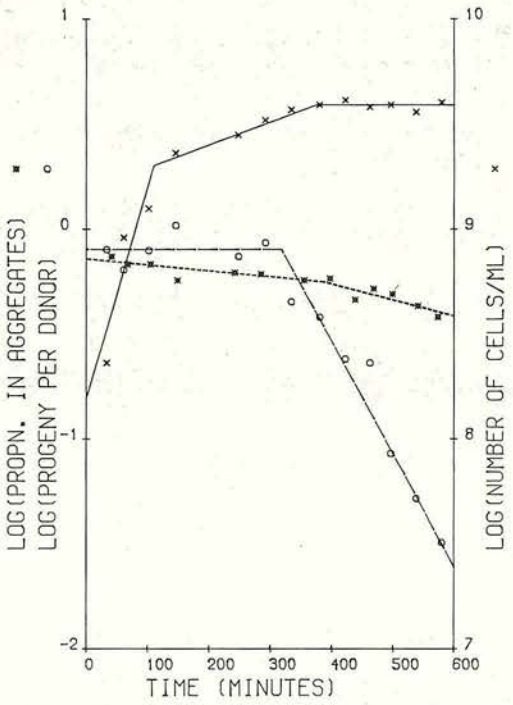

FIG. 2. Effect of growth phase on the progeny-forming ability and aggregate-forming ability of F'*lac* donors. The growth of a culture of JC6582 was followed from exponential phase into stationary phase. At intervals, samples were taken and mated in tubes for 30 min with an excess of an exponentially growing culture of ED2196. The number of Nal^R Lac^+ progeny produced per donor was measured. Samples were also used to measure aggregate-forming ability as described under Materials and Methods. The values shown are not corrected for aggregates in the parent cultures. The proportion of cells in aggregates in the recipient culture remained at about 7.5% throughout the experiment, while the proportion in the donor culture rose from 7.5 to 17%. (×) Donor culture viable count (cells per milliliter); (○) number of progeny per donor in matings; (★) proportion of cells in aggregates in matings.

Thus, retransfer by the recipients does not seem to be greatly affected by the repression system. We were therefore able to use the nonrepressed plasmid F'*lac* to examine the roles of transfer and growth in plasmid spread. F'*lac*, which has a transfer system very similar to that of R100 (Willetts, 1971), was used because well-characterized mutants of it exist (Achtman *et al.*, 1971).

Effect of Growth Phase on Transfer Ability of F'lac

The experiments in this section demonstrate that the donor ability of F'*lac* strains declines rapidly when they enter stationary phase. We followed the growth and transfer ability of a culture of JC6582, an F'*lac* strain, from exponential phase into stationary phase. Figure 2 shows that, when the culture entered stationary phase (at about 300–400 min) the mating ability declined rapidly and had fallen 50-fold by 600 min. We also mixed samples of the culture with the recipient and used a Coulter counter to measure the proportion of cells in aggregates in the mixtures. The proportion of cells in aggregates (Fig. 2) declines much more slowly than the mating ability (from about 75% at 0 min, to about 40% at 600 min). If, by subtraction, we correct for aggregates observed in the parent cultures, we find that the proportion of cells in new aggregates after mixing declined from not less than 70% to not less than 25%, i.e., only about a threefold drop.

The mating ability of JC3272 (F'*lac*) progeny in a mating mixture was determined during a 20-h mating in which JC6582 and JC3272 were mixed at an initial ratio of 1:1000 (Fig. 3a). At intervals we mated samples of the mating mixture with exponential cultures of ED2196 in the presence of phage T6 to pre-

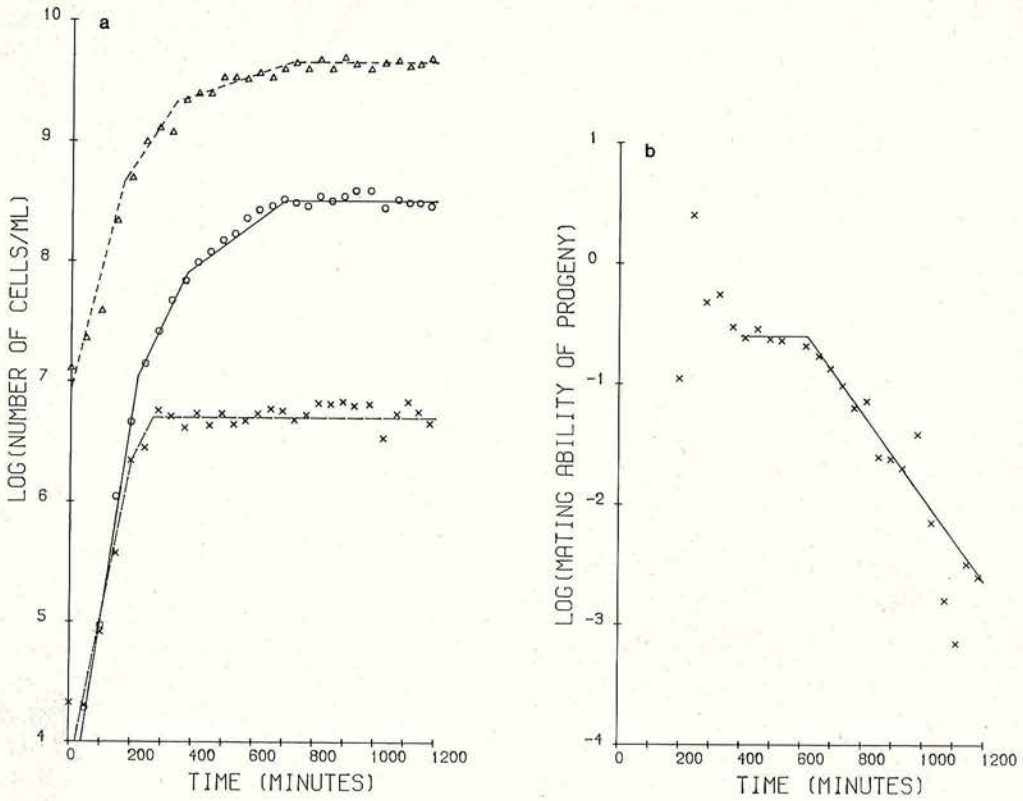

FIG. 3. Exponential cultures of JC6582 and JC3272 were diluted in warm L-broth and mixed to start the mating. (a) The JC6582 donor (×) JC3272 recipient (△) and JC3272 (F′*lac*) (Str^{R}Lac$^+$) progeny (○) concentrations. At intervals, samples were diluted 10^{-4} in warm L-broth and mated for 30 min with exponential cultures of ED2196 (at about 1.5×10^8/ml in the mating tube). The tube contained T6 at a titer above 10^{10} PFU/ml, which prevented progeny formation by the T6^S JC6582. The ratio (×) of ED2196 progeny carrying F′*lac* to the JC3272 (F′*lac*) donors at the end of 30 min matings is shown in b.

vent mating by JC6582. Although there is some scatter among both the early and the later samples, the main point emerges clearly from Fig. 3b, namely, that the mating ability of the JC3272 (F′*lac*) progeny declines rapidly after the recipient enters stationary phase at 700 min.

Importance of Retransfer in Plasmid Spread

To determine the amount of transfer taking place in mating mixtures, we have to know how the progeny growth rate compares with the parental growth rate. We therefore followed the viable count of parallel cultures of JC3272, JC3272 (F′*lac*), and JC3272 (F′*lac traH88*) (see later) from exponential phase into stationary phase. We observed no difference in the growth rate of the three strains. Thus, the fact that in Fig. 3a the rate of increase of progeny numbers (i.e., the slope of the curve) is greater than that of recipient numbers means that transfer must be contributing to the increase of progeny numbers. This transfer is not due to the original donor (JC6582) since these cells enter the stationary phase at 300 min. This was confirmed by matings with ED2196 (the second recipient) in the absence of phage T6 which gave results for the period after 300 min that were very similar to those shown in Fig. 3b.

Further evidence for the importance of retransfer came from experiments in which

TABLE 2

TYPES OF TRANSFER POSSIBLE IN MATING MIXTURES

Plasmid	Phage T6 added?	Possibility of continuing transfer by original donors?	Possibility of retransfer by recipients?
F'*lac*$^+$*traH88*	Yes	No	No
F'*lac*$^+$*traH88*	No	Yes	No
F'*lac*$^+$*tra*$^+$	Yes	No	Yes
F'*lac*$^+$*tra*$^+$	No	Yes	Yes

we compared F'*lac* with F'*lac traH88*, an amber transfer-deficient mutant. This plasmid cannot be retransferred by the JC3272 recipient, which is Su^-, so that all transfers of this plasmid must be by the original (Su^+) donor strain M172. When necessary, we could prevent continuing transfer by the original donors by adding phage T6. The four possible combinations of transfer are shown in Table 2.

We did a control experiment to establish that the efficiency of transfer from the donor strains of the *traH88* plasmid was comparable to that of the *tra*$^+$ plasmid. A mixture of equal volumes of exponential cultures of M172 and JC6582 was mixed with an exponential culture of JC3272 to give a 1:100 ratio of donors:recipients. At 30 and 60 min samples were plated on agar selective for Str^RLac^+ progeny and progeny clones were tested for sensitivity to phage MS2. The $MS2^R$ *traH88* progeny could be distinguished from the $MS2^S$ *tra*$^+$ progeny. This experiment showed that the mutant plasmid was transferred at over 80% of the efficiency of the wild-type plasmid so, as reported by Achtman *et al* (1971), suppression of this mutation is efficient.

Interpretation of the results of 20-h matings with the *traH88* plasmid (Fig. 4) and the *tra*$^+$ plasmid (Fig. 5) is made simpler by the fact that the progeny and recipient growth rates are equal. When retransfer by recipients is prevented (i.e., the *traH88* plasmid is used), the only factor raising the progeny increase rate above the recipient growth rate is continuing transfer by the original donor strain. If this is prevented by addition of phage T6, the progeny increase rate should be equal to the recipient growth rate. This is not very clear in Fig. 4 (+T6) because of sampling errors. However, a similar experiment in which dilutions were chosen to give better estimates of progeny numbers showed that the ratio of progeny to recipients remained constant from 30 min after the addition of phage T6 until the end of the experiment. In contrast, when continuing transfer by the original donor is possible, the progeny increase rate should be greater

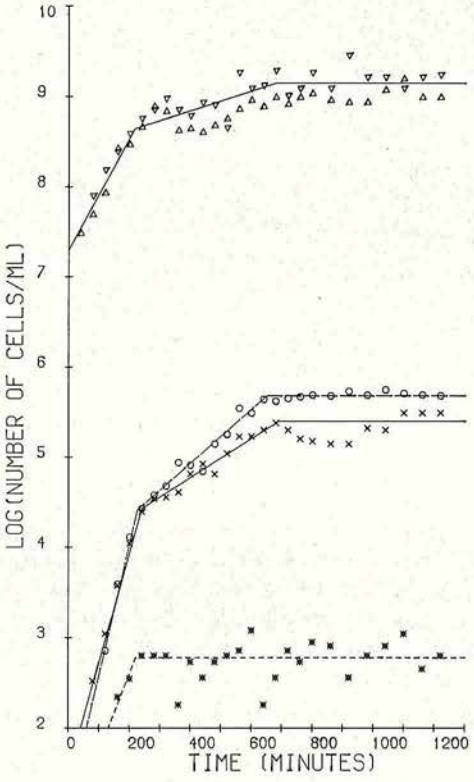

FIG. 4. Mating of M172 (carries F'*lac traH88*) with JC3272. Exponentially growing cultures of the two strains were diluted in warm L-broth and mixed. After 60 min the mating mixture was split into halves and phage T6 was added to one-half to give a titer of about 10^{10} PFU/ml. This reduced the M172 viable count to less than 10/ml for the duration of the experiment. The progeny were selected as Str^RLac^+ cells. M172 donors, × (only for flask without T6); JC3272 recipients, △ (−T6) and ▽ (+T6); JC3272 (F'*lac*) progeny, ○ (−T6) and ★ (+T6).

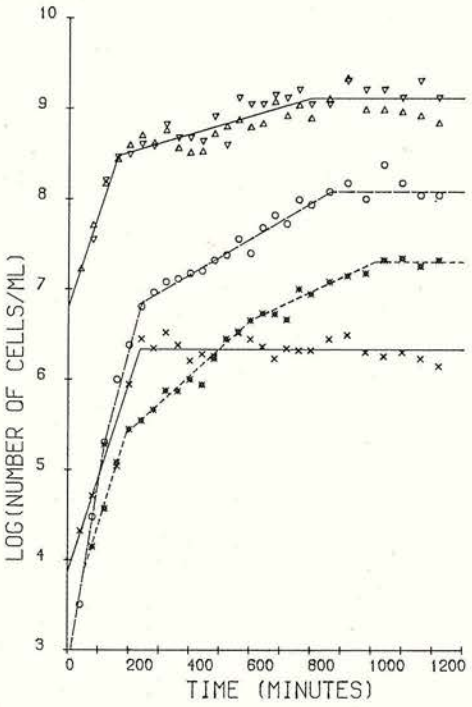

FIG. 5. Mating of JC6582 (carries F'*lac tra*$^+$) with JC3272. The same procedure was used as for the mating in Fig. 4. The phage T6 reduced the JC6582 viable count to less than 10^2/ml for the rest of the experiment. JC6582 donors, × (only for flask without T6); JC3272 recipients, △ (−T6) and ▽ (+T6); JC3272 (F'*lac*) progeny, ○ (−T6) and ★ (+T6).

than the recipient growth rate. However, if the progeny numbers were much greater than the donor numbers, the effects of continuing transfer would become negligible. The effect of allowing continuing transfer from donor cells but not recipient cells is shown in Fig. 4 (−T6). The recipient growth rate is less than the donor growth rate and the donor and recipient cells entered the stationary phase at about the same time (700 min). The progeny numbers rose to about twice the donor numbers. Thus, in the absence of retransfer by recipients, the spread of F'*lac* to a large proportion of the recipient population would only be possible if the proportion of original donor cells in the mating mixture had reached a similar large proportion of the mating mixture.

When retransfer by recipients can occur (i.e., the *tra*$^+$ plasmid is used) there should be some temporal separation of the two types of transfer. In the early stages of the mating mixture retransfer will be negligible (Cullum *et al.*, 1978), but later on, as retransfer becomes important, the relative contribution of continuing transfer by the original donors should become negligible; this is because the ratio of progeny:initial donor strain will become large. In our experiment however, transfer by the original donor had become unimportant from about 300 min because it (but not the recipient strain) had entered stationary phase (Fig. 5; −T6). This is also supported by the fact that the rate of increase of progeny numbers in the half of the culture to which phage T6 was added is very similar to that without T6. However, there was about a 10-fold lower final yield of progeny in the former culture because, when the phage was added (at 60 min), transfer by the original donors was still important. The importance of the length of time during which retransfer can occur is also illustrated by Fig. 5. If the exponential increase in progeny numbers observed in the interval from 300 to 900 min had continued until 1200 min, the final yield of progeny would have been nearly 10 times higher.

Quantitative Estimate of Retransfer

In the experiments shown in Figs. 3a and 5 retransfer by recipients and growth of progeny were both contributing to the increase in progeny numbers. In order to quantitate the effects of growth and transfer, we developed a simple mathematical model which takes into account the considerable time that may elapse between a cell receiving F'*lac* and its being able to retransfer it (about 90 min; Cullum *et al.*, 1978).

We have assumed that recipient concentration does not affect the rate of plasmid transfer as was shown by Cullum *et al.* (1978) for recipient concentrations above 2×10^7 cells/ml. We also assumed that no progeny cell could transfer the plasmid until a time δ after receiving it. A third assumption was

that the rate of plasmid transfer was proportional to the number of competent donors; we used the parameter α to measure the rate per competent donor. The model allows us to determine the value of α under our conditions (see below). In the early stages of a mating mixture discrete "rounds" of transfer might make α time dependent. However, this should be quickly smoothed out by variations between individual cells (Cullum *et al.*, 1978). Any effects that segregation of F− cells by new progeny might have upon this rate per competent donor (Cullum *et al.*, 1978) are included in this parameter α, which is thus the effective progeny-forming rate rather than the plasmid transfer rate.

The rate of increase of progeny numbers is represented as a sum of terms due to growth and retransfer. The growth is represented by $kx(t)$ where k is the exponential growth rate constant and $x(t)$ is the number of plasmid-carrying cells at time t. The retransfer term is the product of α and the number of competent donors at time t. The number of competent donors at time t is the product of the number of progeny cells at time $t-\delta$ and the factor by which these cells have increased by growth in time δ, which is $e^{k\delta}$. Thus, the retransfer term is $\alpha x(t-\delta)e^{k\delta}$ and our model yields the differential equation:

$$\frac{dx}{dt}(t) = kx(t) + \alpha x(t-\delta)e^{k\delta}. \quad (1)$$

The exact solution of this equation depends on the initial conditions, for instance, the inputs of donor and recipient cells. However, we show (see Appendix) that all solutions are approximately of the same form, that is,

$$x(t) = Ae^{(k+\lambda)t}, \quad (2)$$

where A is a constant that depends on the initial conditions and λ is the solution of:

$$\lambda e^{\lambda\delta} = \alpha. \quad (3)$$

Thus the number of progeny increases exponentially with an exponential growth rate that is λ greater than the growth rate; λ depends on the parameters α and δ, not on the initial conditions. If a rate of increase λ is observed, then the larger the lag (δ) is, the greater the mating rate (α) must be to compensate. This is shown by the fact that α increases as δ increases in Eq. (3).

In our experiments using F′*lac* the progeny growth rate is the same as the recipient growth rate; k can therefore be found from the recipient growth rate. λ can then be estimated as the difference between the rate constants for progeny increase and recipient growth.

The main difficulty in estimating α from our results was that we did not know the effect of growth rate on the parameter δ. However, δ must be reasonably small compared to the time scale of the experiments for any retransfer to occur. We assumed a value of δ of 90 min which was that found for exponentially growing cultures (Cullum *et al.*, 1978). If δ were zero, then Eq. (3) would show that α is equal to λ; thus λ forms a lower limit for the estimate of α. We calculated values of α from the experimental results shown in Figs. 3a and 5 (Table 3).

An interesting way of interpreting α is to consider $1/\alpha$ as a mating time. The meaning of $1/\alpha$ can be seen by considering a population of competent donors being mated with a population of recipients growing with the same rate constant (k). If there are N donors at time zero and the number of progeny at time t is $x(t)$, then using assumptions similar to those used for Eq. (1) and ignoring retransfer,

$$\frac{dx}{dt} = \alpha Ne^{kt} + kx, \quad (4)$$

which has the solution:

$$x = \alpha Nte^{kt}. \quad (5)$$

From this it can be seen that progeny numbers x reach the donor number Ne^{kt} at time $1/\alpha$. Table 3 shows the values of the mating time ($1/\alpha$) and the generation time (ln 2)/k calculated from the experimental results shown in Figs. 3a and 5.

TABLE 3

CALCULATION OF MATING RATES

	Figure 3a Calculation based on progeny and recipient numbers in time from 400 to 700 min	Figure 5 Calculation based on progeny and recipient numbers in time from 200 to 900 min
Recipient growth rate constant, k ($\times 10^3$ min^{-1})	2.04	2.30
Rate constant of progeny numbers due to retransfer, λ ($\times 10^3$ min^{-1})	3.23	2.30
Mating rate (α) assuming lag (δ) of 90 min ($\times 10^3$ min^{-1})	4.31	2.83
Generation time, ln2/k (min)	340	301
Mating time ($1/\alpha$) (min)	232	353

We have already found (Cullum *et al.*, 1978) that an exponentially growing F'*lac* donor had a mating time similar to the generation time (both about 30 min). Table 3 shows that in the slow growing cultures the mating time is again similar to the generation time. The generation times shown in Table 3 will not be very accurate because they involve estimating a small increase in bacterial numbers.

The mating ability of samples taken from a mating mixture (Fig. 3b) should also depend on λ. We calculated a value for this mating ability from the value of λ measured in Fig. 3a. We assumed that transferring the cells to a faster growing culture did not affect the rate of mating α, the lag time δ, or the growth rate constant k during the 30 min of mating. The recipient had a generation time of about 30 min. We constructed a differential equation using arguments similar to those used in deriving Eqs. (1) and (4). Substitution of values for k and λ from Table 3 into the solution yielded a predicted value of 0.14 ED2196 (F'*lac*) progeny per JC3272 (F'*lac*) cell during the interval 400–700 min. The observed values had an average of 0.23. The fact that the observed mating efficiency is 50% greater than this predicted value might be explained by transfer to the better growth conditions in the ED2196 culture. We therefore consider that this method of assessing mating ability in a slowly growing culture is reasonably quantitative.

DISCUSSION

We found that we could obtain reproducible mating efficiencies using L-broth. Use of a defined medium might have allowed better control of the time at which cultures enter stationary phase, but the mating efficiency would probably have been lower. In our experiments (Figs. 3 and 5) the fact that the two parent strains enter stationary phase at different times assists the drawing of conclusions on the origin of most of the plasmid transfer.

Comparison of R100 and R100-1 (Fig. 1) shows that repression does not have a great effect on the retransfer of R100 from recipients. This is consistent with the results of Willetts (1974) in which a long time was needed for R100 to suppress F fertility. However, repression does have an important effect on the amount of initial plasmid transfer and this affects final progeny yields. This is shown in Fig. 1; if the ratios of final progeny yield to final numbers of the initial donor strain are examined, it can be seen that there are about 200 times more R100-1 progeny per donor than R100 progeny per donor.

Our experiments with F'*lac* (Figs. 2 and 3) show that transfer ability declines rapidly when donor strains enter stationary phase. Thus, retransfer will only be significant during the period when cell density is high enough for efficient mating (above 10^8 cells/ml; Cullum *et al.*, 1978) and before the re-

cipient strain enters stationary phase. Ozeki *et al.* (1962) found that aeration of mating mixtures greatly reduced the spread of Col I; they interpreted this as being due to the depletion of nutrients during faster growth leading to the rapid cessation of retransfer.

Figure 2 shows that aggregate-forming ability declines more slowly than ability to transfer F'*lac* when donor cells enter stationary phase. Thus Coulter counter measurements of aggregate-forming ability are not always a good indication of the mating ability, defined as the ability to produce F'*lac* progeny. These results would be explained if the presence of a pool of F-pilin in the cell (Beard and Connolly, 1975) allowed pili to be formed and, thus, aggregate formation to occur for a considerable time after expression of other transfer functions had ceased. Curtiss *et al.* (1969) showed that Hfr cells lost the ability to mate after starvation treatment faster than the rate at which pili disappeared. The maximum proportion of cells in aggregates that we observed (77%) is comparable to that observed by Achtman (1975).

In our F'*lac* matings, (Figs. 3 and 5) the recipient and progeny growth rates were the same, so that the contribution of transfer and growth to plasmid spread could easily be assessed. Although growth increases the number of plasmid-carrying cells of the recipient strain, it does not affect the ratio of progeny to recipient cells. Table 3 shows that the rate constant of increase of progeny numbers due to retransfer (λ) is of the same magnitude as the growth rate constant (k). The effect of the lag (δ) on the rate of plasmid increase is fairly small, as can be seen by comparing the rate of mating if a 90-min lag is assumed (α in Table 3) with the value (λ) calculated assuming there was no lag. Thus, the main constraint on the rate of plasmid spread by retransfer during slow growth is the low mating efficiency of recipient cells carrying an established F'*lac*. It is interesting to note that such cells transfer the plasmid about once per 300-min generation; exponentially growing cultures transfer F'*lac* about once per 30-min generation (Cullum *et al.*, 1978). This low mating rate may be a particular property of F'*lac* as the rate of increase of R100 and R100-1 progeny (Fig. 1) is rather higher.

Our results with R100, R100-1, and F'*lac* (Figs. 1, 3, and 5) show that the proportion of recipients in mating mixtures that receive the plasmids is limited because of constraints on initial transfer, the rate of mating, and the time during which transfer can occur. For example, in Fig. 1 over 99% of the increase in the total number of cells carrying R100 during the experiment was due to the increase in numbers of the original donor strain.

It is likely that the opportunities for "epidemic spread" would be even less in nature because many strains are poor recipients in mating (Anderson, 1975) and conditions may not be conducive to transfer. Once an advantageous plasmid–host combination is established by plasmid transfer it can increase in numbers without any significant further transfer. The slow repression of spontaneously derepressed cells in an established population could be necessary, however, to allow sufficient transfer gene expression for a few cells within the population to become competent donors.

APPENDIX

The proof of the behavior of the solutions of Eq. (1) falls into two parts. The first part uses several substitutions to identify the constants λ and A and the second part shows that a remainder term tends to zero.

$$\frac{dx}{dt}(t) = kx(t) + \alpha x(t - \delta)e^{k\delta}. \qquad (1)$$

The first substitution is $x(t) = x(0)P(t)e^{kt}$ so (1) becomes:

$$x(0)\frac{dP}{dt}(t)e^{kt} + kx(0)P(t)e^{kt} = \alpha x(0)P(t - \delta)e^{kt} + kx(0)P(t)e^{kt},$$

$$\frac{dP}{dt}(t) = \alpha P(t - \delta) \quad (6)$$

We then put $P(t) = Q(t)e^{\lambda t}$ where λ is the

solution of $\lambda e^{\lambda\delta} = \alpha$. Substitution into (6) gives:

$$\frac{dQ}{dt}(t) + \lambda(Q(t) - Q(t-\delta)) = 0. \quad (7)$$

Integrating (7) from 0 to t:

$$\{Q(z)\}_0^t + \lambda \int_0^t dzQ(z) - \lambda \int_{t-\delta}^t dzQ(z-\delta) = 0.$$

Substituting $z - \delta$ for z in the second integral gives:

$$Q(t) = (Q(0) + \lambda \int_{-\delta}^0 dzQ(z)) - \lambda \int_{t-\delta}^t dzQ(z). \quad (8)$$

Put a constant $q = (Q(0) + \lambda \int_{-\delta}^0 dzQ(z))/(1 + \lambda\delta)$, put $R(z) = Q(z) - q$, and substitute in (8):

$$R(t) = -\lambda \int_{t-\delta}^t dzR(z). \quad (9)$$

We can use the substitutions above to express x in terms of R:

$$x(t) = x(0)e^{kt}e^{\lambda t}(q + R(t)). \quad (10)$$

If we can show that $R(t) \to 0$ as $t \to \infty$ then we obtain Eq. (2) in which $A = qx(0)$. q is determined by the values of $x(t)$ in times $-\delta$ to 0, which would be determined from the way the mating was set up.

We shall show that $R(t)$ oscillates and that the oscillations become smaller and die away as t becomes large. The first thing to note is that $x(t)$ is a continuous function of t, i.e., there are no instantaneous jumps in the value of $x(t)$. This is necessary biologically and it is also necessary mathematically in order for $dx(t)/dt$ to exist. Thus, $R(t)$ is also continuous as it is the sum and product of $x(t)$ with other continuous functions [Eq. (10)]. As λ is positive, Eq. (9) shows that, if $R(t)$ is positive, then the integral must be negative and hence $R(z)$ must take the value 0 in this interval also. A similar argument shows that this is also true if $R(t)$ is negative. Thus, $R(t)$ oscillates about zero.

$R(t)$ satisfies Eq. (7) [substitute $Q(t) = q + R(t)$]. Multiply equation (7) for R by the integrating factor $e^{\lambda t}$ and integrate from some value a [such that $R(a) = 0$] to t:

$$\int_a^t \left(\frac{dR}{ds}(s)e^{\lambda s} + \lambda e^{\lambda s}R(s)\right)ds - \lambda \int_a^t R(s-\delta)e^{\lambda s}ds = 0.$$

The term inside the first integral is equal to $d(e^{\lambda s}R(s))/ds$ and $R(a) = 0$, so

$$R(t) = \lambda e^{-\lambda t} \int_a^t e^{\lambda s}R(s-\delta)ds. \quad (11)$$

We shall now show that the size of the oscillations decreases geometrically as t increases. We consider t in the time interval $T \leq t \leq T + \delta$ and show that the maximum absolute value of R in this interval $M' = \max|R(t)|$ is less than the maximum value in the time interval $T - 2\delta \leq t \leq T$ which we call M, and in fact we show that $M' \leq (1 - e^{-2\lambda\delta})M$ so that the size of the oscillations in R decreases geometrically.

We choose a in Eq. (11) such that $T - \delta \leq a \leq T$ (this is possible because of the oscillating behavior of R). Then, if $T \leq t \leq T + \delta$ the term $s - \delta$ in Eq. (11) satisfies $T - 2\delta \leq s - \delta \leq T$ so that $|R(s-\delta)| \leq M$. Using elementary properties of absolute values shows:

$$|R(t)| \leq \lambda e^{-\lambda t} \int_a^t e^{\lambda s}|R(s-\delta)|ds.$$

As $|R(s-\delta)| \leq M$ this shows that:

$$|R(t)| \leq \lambda M e^{-\lambda t} \int_a^t e^{\lambda s}ds,$$

and integrating:

$$|R(t)| \leq \lambda M e^{-\lambda t}\{1/\lambda(e^{\lambda s})\}_a^t = M(1 - e^{-\lambda(t-a)}).$$

The choice of a is such that $(t - a) \leq 2\delta$, so that:

$$|R(t)| \leq M(1 - e^{-2\lambda\delta}).$$

As this is true for all values of t in the interval this inequality is also satisfied by the maximum value of M'. Thus, $R(t) \rightarrow 0$ as $t \rightarrow \infty$. Solution of Eq. (1) for various initial conditions showed that the solutions in practice rapidly attained the form of Eq. (2).

ACKNOWLEDGMENTS

We thank the Medical Research Council for support in the form of a training grant (to J.C.) and a project grant (to P.B. and J.F.C.). We thank Helen Grozier for technical assistance. Part of this work was described in a preliminary communication at a meeting at Smolenice, Czechoslovakia, in June 1976.

REFERENCES

ACHTMAN, M. (1975). Mating aggregates in *Escherichia coli* conjugation. *J. Bacteriol.* **123,** 505–515.

ACHTMAN, M., WILLETTS, N., AND CLARK, A. J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *E. coli* by isolation and characterisation of transfer-deficient mutants. *J. Bacteriol.* **106,** 529–538.

ANDERSON, E. S. (1975) Viability of, and transfer of a plasmid from *Escherichia coli* K12 in the human intestine. *Nature (London)* **255,** 502–504.

BEARD, J. P., AND CONNOLLY, J. C. (1975). Detection of a protein, similar to the sex pilus subunit, in the outer membrane of *Escherichia coli* cells carrying a derepressed F-like R factor. *J. Bacteriol.* **122,** 59–65.

BRODA, P. (1975). Transience of the donor state in an *E. coli* K12 strain carrying a repressed R factor. *Mol. Gen. Genet.* **138,** 65–69.

BURMAN, L. G. (1975). Amplification of sex repressor function of one fi$^+$ R-factor during anaerobic growth of *Escherichia coli*. *J. Bacteriol.* **123,** 265–271.

CULLUM, J., AND BRODA, P. (1978). Chromosome transfer and Hfr formation by F in *rec*$^+$ and *recA* strains of *Escherichia coli* K12. Submitted.

CULLUM, J., COLLINS, J. F., AND BRODA, P. (1978). Factors affecting the kinetics of progeny formation with F'*lac* in *Escherichia coli* K12. Plasmid **1,** 536–544.

CURTISS, III, R., CARO, L. G., ALLISON, D. P., AND STALLIONS, D. R. (1969). Early stages of conjugation in *Escherichia coli*. *J. Bacteriol.* **100,** 1091–1104.

LOW, B., AND WOOD, T. H. (1965). A quick and efficient method for interruption of bacterial conjugation. *Genet. Res. Cambridge* **6,** 300–303.

MEYNELL, E., MEYNELL, G. G., AND DATTA, N. (1968). Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. *Bacteriol. Rev.* **32,** 55–83.

OZEKI, H., STOCKER, B. A. D., AND SMITH, S. M. (1962). Transmission of colicinogeny between strains of *Salmonella typhimurium* grown together. *J. Gen. Microbiol.* **28,** 671–687.

STOCKER, B. A. D., SMITH, S. M., AND OZEKI, H. (1963). High infectivity of *Salmonella typhimurium* newly infected by the col I factor. *J. Gen. Microbiol.* **30,** 201–221.

WATANABE, T. (1963). Episome-mediated transfer of drug resistance in *Enterobacteriaceae*. VI. High frequency resistance transfer system in *Escherichia coli*. *J. Bacteriol.* **85,** 788–794.

WILLETTS, N. S. (1971). Plasmid specificity of two proteins required for conjugation in *Escherichia coli* K12. *Nature New Biol.* **230,** 183–185.

WILLETTS, N. S. (1974). The kinetics of inhibition of F'*lac* transfer by R100 in *Escherichia coli*. *Mol. Gen. Genet.* **129,** 123–130.

WILLETTS, N. S., AND FINNEGAN, D. J. (1970). Characteristics of *Escherichia coli* K12 strains carrying both an F prime and an R factor. *Genet. Res. Cambridge* **16,** 113–122.

JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 281-284
0021-9193/79/01-0281/04$02.00/0

Vol. 137, No. 1

Recipient Competence in F′*lac* matings of *Escherichia coli* K-12

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Received for publication 18 October 1978

We studied recipient mating ability in the presence of excess F′*lac* donors. Ninety-five percent of recipients were able to receive F′*lac* in 30-min matings. Competition between an F′*lac* donor and an F′*lac traI* donor, which mobilized a ColE1 derivative (pML2), showed that each recipient mated with an average of two to three donors in 30 min. Experiments in which the competing donor was added at different times showed that some competition occurred throughout the 30-min mating period, which suggested that aggregate formation was spread over this time.

Many experiments with F′ and Hfr matings have used approximately equal numbers of donors and recipients. This introduces complications in the quantitative interpretation of matings because it is not known which parent is the limiting factor. We previously studied some aspects of donor behavior in F′*lac* matings in which the recipients were in excess (6). Here, we describe experiments designed to investigate recipient behavior in matings in which the donors are in excess.

We consider three aspects of recipient competence. (i) We considered the proportion of recipients able to mate with donors. Walmsley investigated pair formation in short (7-min) Hfr matings (10). He concluded that only about 30% of recipients were competent to form pairs. However, he used a correction which assumed that mating followed bimolecular reaction kinetics; this assumption is not supported by the results of Collins and Broda (5) (see reference 6). (ii) We considered the number of donors mating with each recipient. The demonstration of mating aggregates containing many cells (1) suggested that multiple matings might occur frequently. However, multiple mating of donors with several recipients was infrequent in the case of F′*lac* donors (6) and Hfr donors (4). (iii) We also considered the spread in times at which recipients are capable of forming aggregates. This would be in addition to the spread in time between aggregate formation and plasmid transfer (8).

The proportion of recipients able to mate with donors was estimated in matings where donors were in excess. If there is a restriction on the number of donors that can mate with any recipient, then there should be competition between different donor strains. However, we could not use recovery of recipient cells containing two different F′ plasmids as an assay of multiple mating because of the problem of incompatibility. Instead, we used an F′*lac traI* strain that can form aggregates but not transfer the plasmid (2). The effect of different ratios of the F′*lac tra*$^+$ and F′*lac traI* strain on the yield of Lac$^+$ progeny allowed us to estimate the number of donors mating with each recipient. In addition, adding the competitor at different times after the start of mating allowed us to study the temporal spread in aggregate formation.

MATERIALS AND METHODS

Bacterial strains are shown in Table 1. Media, growth conditions, and tube matings are described elsewhere (6). Kanamycin (Winthrop) was used at a final concentration of 50 μg/ml. Aggregate formation was measured with a Coulter Counter as described elsewhere (7).

Tube matings were started by mixing 1 ml of donor culture with 1 ml of a culture of ED2196; both parent cultures were growing exponentially. The proportion of Lac$^+$ recipients was scored by plating on lactose tetrazolium agar containing nalidixic acid.

Estimation of recipient mating profile from competition experiments. We assumed that every recipient had a certain number of mating sites and that the two donors JC6582 and ED2510 competed equally for these sites. We also assumed that if JC6582 donors occupied one or more sites on the recipient then the recipient became Lac$^+$. If there is a proportion q of ED2510 donors (and therefore $1 - q$ of JC6582 donors), then a recipient with n mating sites will only remain Lac$^-$ if all sites are occupied with ED2510; this outcome has a probability of q^n if all donors act independently. If for $n = 0, 1, 2, \ldots$ there is a proportion p_n of recipients that have exactly n mating sites, the total proportion of recipients in the population that remain Lac$^-$ will be $\sum_{n=0}^{\infty} P_n q^n$. We found (see

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TABLE 1. *Bacterial strains*

Strain	Plasmid carried	Host strain[a]
JC6582	F′*lac*	JC6255
ED2510	F′*lac traI65*, pML2	JC6256
ED2196	F⁻	ED2196
ED2525	F′*lac traI40*	JC3272
ED2526	F′*lac traA*	JC3272

[a] The F′*lac* plasmids are isogenic with each other and described elsewhere (2). Achtman et al. (2) also describe the "host" strains, i.e., the isogenic plasmid-free strains. ED2196 is resistant to nalidixic acid, and the other strains are sensitive. All strains were kindly provided by N. Willetts, except ED2510, which was constructed by P1 transduction of pML2 (kindly provided by D. Finnegan) into the F′*lac traI65* strain.

below) that a Poisson distribution for the number of donors mating with each recipient gave a good fit to the data; i.e., $P_n = \lambda^n e^{-\lambda}/n!$ where λ is the mean number of donors mating with each recipient. This gives the proportion of the recipients that remain Lac⁻ as $e^{\lambda}(q - 1)$. If it is assumed that a proportion (α) of the ED2510 donors that bind transfer pML2 to the recipient, then similar calculations show that the proportion of recipients that remain Kan^s is $e^{-\alpha\lambda q}$.

RESULTS

Proportion of competent recipients. In matings at a cell density of above 5×10^7 cells per ml with one parent in excess, we found that the number of progeny per minority parent reached a plateau after 30 min. We therefore used 30-min matings for further experiments.

We first wanted to find the proportion of recipients that were able to receive F′*lac*. Matings were performed with a constant high concentration of donor cells and with various donor/recipient ratios. At high donor/recipient ratios (above 10:1) the great majority (90% to 95%) of recipients were capable of receiving F′*lac*. A lower proportion (46%) received F′*lac* in 1:1 matings which presumably reflects a shortage of donors. The recipient viable count showed no depression due to "lethal zygosis," in agreement with Skurray et al. (9). In subsequent matings, we therefore used a donor concentration of 2×10^8 cells per ml and a recipient concentration of 2×10^4 cells per ml.

Number of donors mating with each recipient. If each recipient is only capable of aggregating with a limited number of donor cells, the addition of an F′*lac traI* strain that can form aggregates but not transfer F′*lac* (2) should reduce the number of progeny formed by an F′*lac tra*⁺ strain in a mating mixture. Table 2 shows that this is indeed the case. On the other hand, an F′*lac traA* mutant, which does not form aggregates with recipients, has little effect on progeny formation (Table 2).

Table 2 also shows that the aggregate-forming ability of the F′*lac traI* strain ED2510, as measured by a Coulter Counter, was identical with that of the F′*lac tra*⁺ strain (JC6582). We have therefore made the simplifying assumption that these strains are equal competitors with respect to aggregate formation. Using this simplifying assumption, we performed competition experiments to estimate the number of donors with which each recipient was capable of mating. The more donors with which a recipient can mate, the less sensitive progeny formation will be to inhibition by competing donor strains. We can therefore determine the number of donors with which each recipient can mate from the inhibitory effect of different ratios of competing donors upon the F′*lac* progeny yield (see above and Fig. 1). A Poisson distribution of the number of donors mating with each recipient gave a good fit to our results. We assumed that aggregation with one or more JC6582 cells resulted in the recipient receiving F′*lac* because every F′*lac* donor can mate with one recipient in 30 min (6). Figure 1 shows the theoretical curve corresponding to a mean of 2.65 donors per recipient. Thus, recipients are capable of mating with two to three donors, on average, in 30 min.

The strain ED2510 also contains a ColE1 derivative, pML2 (Kan^r), which is mobilized by F′*lac traI* (3). Transfer of pML2 proved useful

TABLE 2. *Inhibition of JC6582 matings and aggregate-forming ability*

Strain	F′*lac* carried	Other plasmid carried	JC6582 mating inhibition[a]	% Cells in aggregates in mating mixtures with ED2196[b]	% Cells in aggregates in parent cultures[b]
JC6582	*tra*⁺		0	62	23
ED2525	*traI*		0.18	62	30
ED2526	*traA*		0.05	15	16
ED2510	*traI*	pML2	0.21	62	23
ED2196	F⁻				14

[a] The quantity shown is one minus the relative proportion of recipients that become Lac⁺ in 30-min matings of a 1:1 mixture of the two donors with an equal volume of ED2196 culture diluted to about 2×10^4 cells per ml. The relative proportion is derived by dividing the observed proportion by the proportion that became Lac⁺ in control matings of JC6582 with ED2196.

[b] A Coulter Counter was used to measure the proportion of cells in aggregates as described before (7). The culture being tested was blended to break up aggregates, and the blended cultures were compared with an unblended culture. The matings were 1:1 matings of donor and recipient cultures at the same optical density for 30 min.

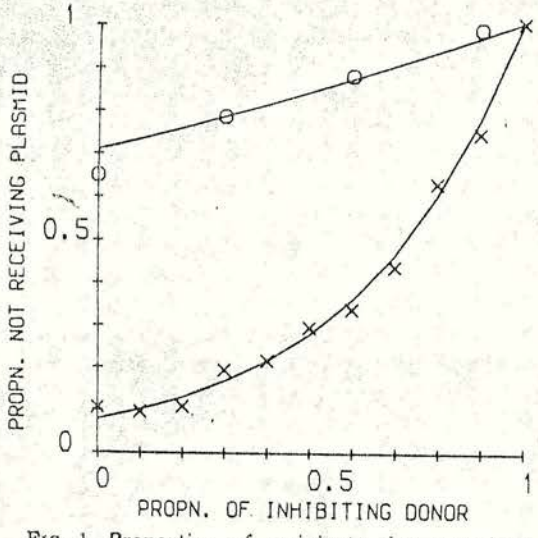

FIG. 1. *Proportion of recipients that remained Lac⁻ when mated with JC6582:ED2510 mixture plotted against the proportion of ED2510 in the donor mixture* (×). *The theoretical curve corresponds to a Poisson distribution (mean, 2.65) of the number of donors mating with each recipient. Proportion of recipients that remained Kan^s after mating with an ED2510:JC6582 mixture plotted against the proportion of JC6582 in the donor mixture* (○). *The theoretical curve corresponds to a Poisson distribution (mean, 2.65) of the number of donors mating with each recipient and an efficiency of Kan^r progeny formation per aggregated ED2510 donor of 0.13.*

as an independent assay of the involvement of ED2510 in mating complexes. The fertility of ED2510 for pML2 transfer was lower than that of JC6582 for F′*lac* transfer; in 30-min matings with an excess of recipients, only 0.1 to 0.2 Kan^r progeny per donor were produced in comparison with about 1 Lac$^+$ progeny per donor in parallel JC6582 matings. If deficiency in aggregation were the cause of this lower fertility, then ED2510 matings with an excess of donors should result in a high proportion of recipients becoming Kan^r. However, in matings with 2×10^8 ED2510 donors per ml and 2×10^4 recipients per ml, only 35% of recipients became Kan^r. This suggests that there is a failure in progeny formation after aggregation rather than a failure in aggregation. This agrees with the Coulter Counter measurements (Table 2) that showed that aggregation by ED2510 was identical with that by JC6582. We also measured the proportion of recipients that became Kan^r in matings with different ratios of ED2510 and the inhibiting donor JC6582 (Fig. 1). The experiment in which the proportion of Lac$^+$ progeny was measured for different donor ratios (Fig. 1) gave the number of donors mating with each recipient. Use of these data gave theoretical curves (see above) for the proportion of recipients that become Kan^r at different donor ratios. If it is assumed that the efficiency of ED2510 mating is 0.13 pML2 transfers per aggregated ED2510 cell, then this theory gives a curve in reasonable agreement with the results (Fig. 1).

Time of aggregate formation. We also wanted to examine the time of aggregate formation. If all mating aggregates were formed rapidly and the observed kinetics of progeny formation were due to the spread in times of transfer after aggregation (8), then the production of F′*lac* progeny in JC6582 matings should quickly become insensitive to addition of ED2510. To test this, we performed a series of parallel 30-min matings of JC6582 with ED2196. ED2510 was added to each of the matings at a different time, and the proportion of recipients that became Lac$^+$ after a 30-min mating was measured.

There was some inhibition throughout the 30-min mating time which showed that there was a spread in times of aggregation. Addition of the competing strain after 20 min still produced a 20% drop in progeny yield. It is difficult to interpret these results quantitatively, because it is not known whether the number of donors which can mate with each recipient is invariant or whether it depends on the time at which aggregate formation can occur.

DISCUSSION

We found that practically all recipients are capable of receiving F′*lac* in 30-min matings. At first sight this is in contrast to the conclusion of Walmsley (10) (for Hfr matings), who found that only about 30% of recipients seemed competent to form aggregates. However, part of the difference might be accounted for by the short time (7 min) of his matings, because we found that there appeared to be a spread in times in which recipients could form aggregates. Thus, in short matings some recipients would be unable to form aggregates although they would mate successfully in 30 min. It is not clear whether the 5% of colonies that remain Lac$^-$ are descended from cells that have never mated or from F$^-$ segregants from cells that have mated (6, 8).

We deduced from the results shown in Fig. 1 that each recipient is capable of mating with an average of two to three donors in 30 min. This is in contrast to the behavior of donors (6) which can only mate once in 30 min. This helps to explain why only 45% of recipients receive F′*lac* in 1:1 donor/recipient matings—some recipients mate several times and "use up" donors so that other recipients cannot mate. This effect would

be accentuated by the spread in times that recipients can mate.

ACKNOWLEDGEMENT

We thank the Medical Research Council for a training grant (to J.C.) and a project grant (to P.B.).

LITERATURE CITED

1. **Achtman, M.** 1975. Mating aggregates in *Escherichia coli* conjugation. J. Bacteriol. 123:505–515.
2. **Achtman, M., N. Willetts, and A. J. Clark:** 1971. Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterisation of transfer-deficient mutants. J. Bacteriol. 106:529–538.
3. **Alfaro, G., and N. Willetts.** 1972. The relationship between the transfer systems of some bacterial plasmids. Genet. Res. 20:279–289.
4. **Broda, P., and J. F. Collins.** 1978. Role of simple and complex aggregates in *Escherichia coli* Hfr × F⁻ matings. Genet. Res. 31:167–175.
5. **Collins, J. F., and P. Broda.** 1975. Motility, diffusion and cell concentration affect pair formation in *Escherichia coli*. Nature (London) 258:722–723.
6. **Cullum, J., J. F. Collins, and P. Broda.** 1978. Factors affecting the kinetics of progeny formation with F′*lac* in *Escherichia coli* K12. Plasmid 1:536–544.
7. **Cullum, J., J. F. Collins, and P. Broda.** 1978. The spread of plasmids in model populations of *Escherichia coli* K12. Plasmid 1:545–556.
8. **De Haan, P. G., and A. H. Stouthamer.** 1963. F-prime transfer and multiplication of sexduced cells. Genet. Res. 4:30–41.
9. **Skurray, R. A., N. Willetts, and P. Reeves.** 1976. Effect of *tra* mutations on F factor-specified immunity to lethal zygosis. Mol. Gen. Genet. 146:161–165.
10. **Walmsley, R. H.** 1973. Physical assay of competence for specific mating-pair formation in *Escherichia coli*. J. Bacteriol. 114:144–151.

JOURNAL OF BACTERIOLOGY, June 1977, p. 1274-1280
Vol. 130, No. 3

Printed in U.S.A.

Molecular Sizes and Relationships of TOL Plasmids in *Pseudomonas*

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Received for publication 8 February 1977

Plasmid deoxyribonucleic acid was isolated from thirteen *Pseudomonas* strains judged on genetic criteria to carry plasmids coding for the degradation of toluene and *m*- and *p*-xylenes (TOL plasmids). Most strains carried a single species, but two strains carried two size classes, and cells of a third strain contained plasmids ranging in size from 25×10^6 to 202×10^6 daltons. Some plasmids could be transformed into a *Pseudomonas putida* strain to yield Tol^+ progeny. Plasmids from 5 of the 13 strains were indistinguishable on the basis of size and gel pattern of fragments after endonuclease digestion.

Williams and Worsey (17) have described strains of *Pseudomonas* isolated independently from soil after enrichment culture in which *m*-toluate was the sole carbon source. These strains can also degrade toluene via benzoate and *m*- and *p*-xylene via *m*- and *p*-toluate (the Tol function). Genetic evidence was presented that in these strains this degradative function was plasmid mediated, as had previously been shown in *Pseudomonas putida* (*arvilla*) mt-2 (16, 18, 19). In this communication we (i) describe the molecular characterization of plasmids from these strains, (ii) present evidence that in most cases the presence of the plasmid is correlated with the possession of the Tol function, and (iii) describe experiments that test whether the plasmids from the different isolates are related to each other.

MATERIALS AND METHODS

Bacterial strains. The toluene- and xylene-utilizing bacteria used in this investigation have been described previously (17). The recipient for transformation, strain AC34, was kindly provided by A. M. Chakrabarty. It is an adenine-requiring derivative of *Pseudomonas putida* strain PgGl (11). Strains of *Escherichia coli* harboring the plasmids RP1 and ColE1 were obtained from G. Humphreys and N. S. Willetts, respectively.

Materials. Nitrocellulose (DHX 30/50) was obtained from the Nobel Division of I.C.I. Ltd., Stevenston, Ayrshire, Scotland. Egg-white lysozyme (grade I, lyophilized) and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo. All other materials were obtained from sources previously described (15).

Culture conditions and determination of growth rates. When it was necessary to select for and to ensure retention of the Tol^+ phenotype in bacterial strains, a minimal salts medium containing *m*-toluate as sole carbon source was used. Liquid medium was made by the addition of sterile 1 M *m*-toluate to 80 ml of 4×-concentrated M9 medium (1) to give a final concentration of 10 mM. This mixture was added to 300 ml of sterile water to which had also been added 0.5 ml of stock salts solution (3), 0.25 ml of 1 M $MgSO_4$, and 0.25 ml of 36 mM $FeSO_4$. This method of preparation causes minimal precipitation of phosphates and hydroxides of heavy metals and avoids the need to use an organic chelating agent. This medium was solidified by the inclusion of 1.5% New Zealand agar to give selective plates and slants on which stock cultures were maintained. Other prototrophic strains were maintained on minimal agar slopes containing either succinate or benzoate as the carbon source. Auxotrophic strains were maintained on L-broth agar slopes. L-broth contains 10 g of tryptone (Difco), 5 g of NaCl, and 5 g of yeast extract (Difco) per liter. Bacterial buffer contains 3 g of KH_2PO_4, 7 g of Na_2HPO_4, 4 g of NaCl, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter.

For the isolation of degradative plasmid deoxyribonucleic acid (DNA), cell cultures were grown to stationary phase in 25 ml of selective medium containing *m*-toluate. Such cultures were inoculated into 1-liter volumes of Spizizen minimal medium (14) containing 0.2% glucose, in 2-liter conical flasks. Cell density was measured turbidimetrically with a Klett-Summerson colorimeter with a red filter. Bacterial cultures were grown in rotary shakers at 30°C for *Pseudomonas* strains and at 37°C for *E. coli* strains.

Isolation of plasmid DNA. Phage λ DNA was prepared as described (9). ColE1 and RP1 DNA were prepared from *E. coli* strains ED678 (obtained by the transfer of ColE1 into strain JC 6256 [2]) and UB 1139 (5), respectively, as described previously (15). Chloramphenicol was added to exponentially grow-

ing cultures (3×10^8 cells/ml) of the former strain to a final concentration of 60 μg/ml in order to increase the number of copies of ColE1 DNA per cell (7).

Isolation of degradative plasmid DNA. In repeated attempts, we were unable to isolate degradative plasmid DNA as covalently closed circular molecules in an ethidium bromide-CsCl density gradient after a clearing spin had been used to remove chromosomal DNA. After the finding (11) that such plasmid DNA could be isolated after removal of chromosomal DNA by alkaline denaturation and adsorption to nitrocellulose, the following modification of the method of plasmid isolation (13) was used. Using this method, a single person can prepare plasmid DNA from at least six strains simultaneously, the main work of the preparation occurring during 4 of the working days of 1 week.

One-liter batches of bacterial cultures were grown to late exponential phase (approximately 10^9 cells/ml or 180 Klett units) in Spizizen minimal medium and harvested by centrifugation at 4°C. The pellet was resuspended in 21 ml of cold sucrose solution [25% sucrose in 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0] and stored in this form at −20°C. After thawing, spheroplasts were formed by adding lysozyme solution (3 ml of freshly prepared solution at 10 mg/ml in 0.25 M Tris-hydrochloride, pH 8.0) and shaking at 37°C for 2 min, followed by storage on ice. After 5 min, 13 ml of 0.25 M ethylenediaminetetraacetic acid (EDTA) solution (sodium salt, pH 8.0) was added, and after a further 5 min the cells were lysed by adding 27 ml of an aqueous solution of 2% Triton X-100, 0.05 M Tris-hydrochloride (pH 8.0), and 0.0625 M EDTA. After allowing up to 1 h for lysis, the DNA in the resulting viscous solution (approximately 60 ml) was sheared by slow passage through the Luer nozzle of a 50-ml disposable syringe for 45 to 60 s. The sheared lysate was denatured (to pH 11.5) by the addition of 2 ml of freshly made 4 N NaOH solution (or a solution that had been stored in a stoppered bottle) while stirring with both a magnetic follower and glass rod. When the mixture appeared homogeneous, the glass electrode of a pH meter was used to ascertain the homogeneity of pH (i.e., that there were no pockets of extreme pH). The solution was held at pH 11.5 for 60 s while being mixed gently; the pH was then rapidly restored to between 8.0 and 8.5 by the addition of 25 ml of saturated (at room temperature) Tris-hydrochloride solution, pH 7.0. The single-stranded DNA from denatured chromosome and open circular plasmid molecules was removed by adsorption to nitrocellulose. Approximately 20 g (wet weight) of nitrocellulose, which had been ground to fibrous mats with a porcelain mortar and pestle and washed extensively with distilled water, was sprinkled into the DNA solution and gently rotated with it for 1 h at 4°C. The nitrocellulose with the associated single-stranded DNA was removed by centrifugation at 5,000 rpm for 10 min at 4°C; this extraction step was then repeated by a further addition of nitrocellulose to the supernatant. After centrifugation, each solution was filtered through glass wool into a polycarbonate centrifuge bottle, underlayered with 4 ml of saturated CsCl (at room temperature) in TES buffer (0.05 M Tris-hydrochloride, 0.005 M EDTA, and 0.05 M NaCl, pH 8.0), and centrifuged (for 15 to 20 h) at 18,000 rpm ($32,000 \times g$ at r_{av}) in a fixed-angle type 21 rotor (Beckman-Spinco) at 4°C after the centrifuge bottle was completely filled by careful overlayering with distilled water. After gentle aspiration of the upper 90 ml, the lowest 9 ml of each tube was collected, gently mixed, and filtered through a glass wool plug into polyallomer centrifuge tubes. Solid CsCl was added to bring the refractive index to 1.402, and the tubes were each filled to within 1 cm of the top with CsCl solution of refractive index 1.402. Last, 0.2 ml of ethidium bromide solution (20 mg/ml in water) was added and gently mixed with the tube contents by inversion. From this step onward, the tubes were protected from light whenever possible to minimize light-activated dye nicking of covalently closed DNA molecules. The centrifuge tubes were overlayered with liquid paraffin and centrifuged at 40,000 rpm ($100,000 \times g$ at r_{av}) for 40 h in a fixed-angle type 50 Ti rotor (Beckman-Spinco) at 15°C. The DNA banded within the density gradient to form (i) an upper viscous band comprising linear chromosomal and open circular plasmid DNA and (ii) a lower band comprising covalently closed circular plasmid DNA. These were apparent under long-wavelength ultraviolet illumination and, if present in high yield, were also visible against a white background in daylight. The DNA bands were collected by aspiration into a Pasteur pipette connected to a 1-ml disposable syringe by a cut rubber teat. The upper band was removed first and discarded. When necessary, the material from the plasmid band was pooled and recentrifuged to concentrate the plasmid DNA and to obtain a purer preparation. However, it was generally found that a single ethidium bromide-CsCl density gradient centrifugation step was adequate to isolate plasmid DNA in a sufficiently pure form to allow subsequent characterization by electron microscopy and agarose electrophoresis after endonuclease digestion.

Ethidium bromide was removed from the plasmid preparation by gentle extraction with propan-2-ol (presaturated with CsCl solution) followed by dialysis against the sodium form of Dowex 50W-XB resin in 50 ml of buffer (0.8 M NaCl, 0.05 M Tris-hydrochloride, 0.01 M EDTA, pH 8.0) (13). The DNA solution was finally dialyzed against several changes of TE buffer (0.01 M Tris-hydrochloride and 0.001 M EDTA, pH 7.2) before storage at 4°C. The DNA concentration was determined spectrophotometrically, assuming that an absorbance at 260 nm of 1.0 corresponds to 50 μg/ml. Yields of 30 to 50 μg of plasmid DNA per preparation were obtained.

Transformation. A scaled-down version of a published transformation procedure (6) was used, with strain AC34 as recipient. A 50-μl amount of chilled calcium-treated cells was added to a mixture of 20 μl of plasmid DNA, containing approximately 0.5 μg of DNA, and 5 μl of 0.5 M $CaCl_2$ at 0°C, mixed, and stored on ice for 60 min. After the heat-pulse step (42°C, 5 min), the cell suspension was kept on ice for at least 30 min, after which a 10-μl sample was removed with a micropipette, mixed with 0.1 ml

of bacterial buffer, and plated out onto selective medium, containing 10 mM *m*-toluate and adenine (50 μg/ml), for the detection of transformants.

The remainder of the cell suspension was mixed with 10 volumes of L-broth and shaken at 30°C for 3 h. The cells were then harvested by centrifugation and resuspended to the same density in bacterial buffer. Samples of 0.1 and 0.2 ml were plated out onto the selective agar plates. Viable counts were made at all stages of the transformation procedure on L-broth agar.

Electron microscopy. Plasmid DNA was visualized by the formamide method (8).

Restriction endonuclease digestion of plasmid DNA. Digestion was carried out in silicone-coated (Repelcote; Hopkins and Williams) small tubes (12.5 by 50 mm) sealed with Parafilm (Gallenkamp) at 37°C for up to 4 h, depending upon the activity of the enzyme preparation. DNA preparations in TE buffer containing approximately 1 μg of DNA were each dried down to 20 μl in a vacuum desiccator over concentrated H_2SO_4 at a reduced pressure of 12 mm of Hg. The digestion mixture was adjusted to 10 mM Tris-hydrochloride (pH 7.5), 10 mM 2-mercaptoethanol, 100 mM NaCl, and an excess of 10 mM $MgCl_2$ over the EDTA concentration. Endonuclease *Eco*RI, prepared by the method of R. Yoshimori (Ph.D. dissertation, University of California, San Francisco, 1971) in 50% glycerol, was added in requisite amounts, usually 1 to 2 μl, to the digestion mixture. Endonuclease *Xma* I, purified from *Xanthomonas malvacearum* and which cleaves the DNA sequence

5′ - C↓C C G G G - 3′
3′ - G G G C C↑C - 5′

(S. Endow and R. J. Roberts, manuscript submitted for publication), was used in a similar manner.

After incubation, the reaction was terminated by heating in a water bath at 70°C for 10 min, after which the tubes were rapidly chilled in ice water; this prevents the cohesive ends of λ DNA from reannealing. The contents of each tube were mixed with 5 μl of loading mixture, comprising 10% Ficoll (Pharmacia) and 0.04% bromophenol blue, on polythene sheeting, and the liquid volume was reduced to about 10 μl by evaporation in a vacuum desiccator before the concentrated solution was loaded into the sample wells of the agarose slab gel.

Agarose gel electrophoresis. Electrophoresis was carried out on vertical slab gels (1% agarose) as previously described (15) except that ethidium bromide was omitted from the gel buffer and reservoir buffer and the lowest 3 cm of the slab gel was formed from 3% agarose in electrophoresis buffer to prevent gel slippage. After electrophoresis at a constant voltage of 150 V for 15 to 20 h, when the blue tracking dye had traveled about three-quarters of the length of the plate, the agarose gel was removed from the glass plates and stained for 30 min in aqueous ethidium bromide solution (2 μg/ml), after which the gel was washed in water for up to 3 h before photography. The gels were photographed against a black polythene sheeting background under short-wavelength ultraviolet illumination through a ×4 red filter onto Ilford FP4 film.

Densitometry. Densitometric scans of photographic films of gels were made on a MK.III double-beam recording microdensitometer (Joyce, Loebl and Co. Ltd., Gateshead, England).

RESULTS

Isolation and size determination of plasmid DNA. The procedure described above yielded from each of the strains examined DNA that gave a discrete band of presumptive plasmid DNA in ethidium bromide-CsCl density gradient centrifugation. This DNA was examined by electron microscopy. The preparations from most strains contained only a single size class of circular molecules. From each such preparation six molecules were measured, using ColE1 or RP1 molecules as internal standards (Table 1). Strains MT1 and MT3 yielded plasmids that fell into two size classes, and another strain, MT14, gave circular molecules of a number of different sizes (Table 2). The molecules from MT15 and MT19, and some of those from MT14, were very large. It has so far not been possible to obtain circular molecules from the presumptive plasmid DNA preparation from strain MT20 under these conditions. The possible reasons for this will be discussed in another communication.

Gel electrophoresis of endonuclease digests of plasmid DNA. Since several of the plasmids were very similar in size (about 77 megadaltons [Md]), it was possible that they were related. One test of identity is by digestion with site-specific endonucleases, followed by electrophoresis on agarose gels. Similar patterns of frag-

TABLE 1. *Sizes of plasmid molecules observed*[a]

P. putida strain	Plasmids size (Md ± standard error)	Name of plasmid
mt-2	78.1 ± 1.1	pWW0
MT16	81.3 ± 2.5	pWW16
MT17	75.0 ± 0.6	pWW17
MT18	75.6 ± 0.9	pWW18
MT21	74.4 ± 0.8	pWW21
MT1	52.0 ± 0.4	pWW1
	4.8 ± 0.1	pWW2
MT3	79.9 ± 0.8	pWW3
	4.0 ± 0.1	pWW4
MT5	103.3 ± 4.9	pWW5
MT12	75.1 ± 1.4	pWW12
MT13	75.7 ± 1.4	pWW13
MT14	25–202	
MT15	169.1 ± 2.6	pWW15
MT19	151.5 ± 2.7	pWW19

[a] Grids were first made without any internal standard. Then new grids were made including ColE1 (taken as 4.2 Md [4]) or RP1 (determined using ColE1, as 38.9 ± 0.5 Md) as internal standards.

TABLE 2. *Size distribution of circular plasmid molecules from strain MT14*[a]

Size class (Md ± standard error)	No. of molecules involved
25.2 ± 0.5	6
39.5 ± 0.8	8
60	3
68	3
76	1
202.5 ± 1.5	—[b]

[a] All such molecules on a given grid were photographed and measured. ColE1 was used as an internal standard.

[b] A total of 20 very large molecules were identified. The value given is for the seven, chosen at random, that were measured.

ments are evidence of relatedness (15). The results of such experiments with endonuclease *Eco*RI are presented in Fig. 1.

By this criterion, the plasmids with sizes of about 77 Md fell into two groups, typified by those carried by strains *P. putida* mt-2 and MT12 (Fig. 1). Digestion of the first set with another endonuclease, *Xma* I, also gave indistinguishable patterns. We conclude that this type of plasmid is widely distributed in nature. Strains MT12 and MT13 were isolated from the same enrichment culture, but could be distinguished by their morphology (17). Therefore a given plasmid can be present in different and distinguishable *Pseudomonas* species.

The plasmid DNA from strains MT1, MT3, MT5, and MT19 gave unique patterns, as expected from the size determinations, but seemed to have common bands (Fig. 2). The possibility that these plasmids are related is currently being tested by heteroduplex and annealing methods.

Densitometry traces of the gels showed that the *Eco*RI digest of the *P. putida* mt-2 plasmid DNA gave about 29 bands, on the assumption that peaks representing twice the unit amount of DNA represented coincident bands (Fig. 3). However, it is not excluded that some such double-large peaks represent duplications of particular regions. Comparison of the mobilities of these fragments with those of fragments of known size derived from a concomitant digestion of phage λ DNA indicated that the sum of the sizes of the fragments was about 75 Md. Since this was very similar to the size of the plasmid DNA as determined by electron microscopy, we conclude that this indeed represents a single plasmid species. Similar arguments apply to the other plasmid preparations containing a single size class.

Correlation of plasmid species with Tol⁺ phenotype. Transformation provides the most rigorous demonstration that a given plasmid species is responsible for a given phenotype. We have transformed plasmid DNA from *P. putida* mt-2 into a genetically marked plasmid-free strain, AC34. Our frequency of transformation (equivalent to 30 colonies per μg of DNA) was lower than that observed for the TOL plasmid

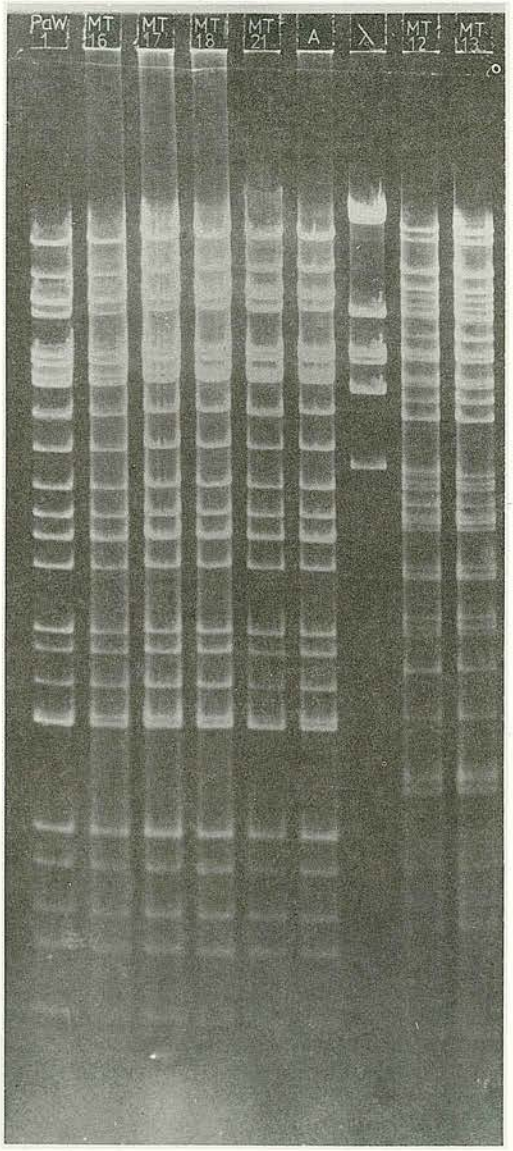

FIG. 1. *Agarose gel electrophoresis of fragments of plasmid DNA generated by EcoRI digestion. The track marked "A" is of plasmid DNA from a transformant clone (see text), and a λ digest is included to calibrate the sizes of the fragments.*

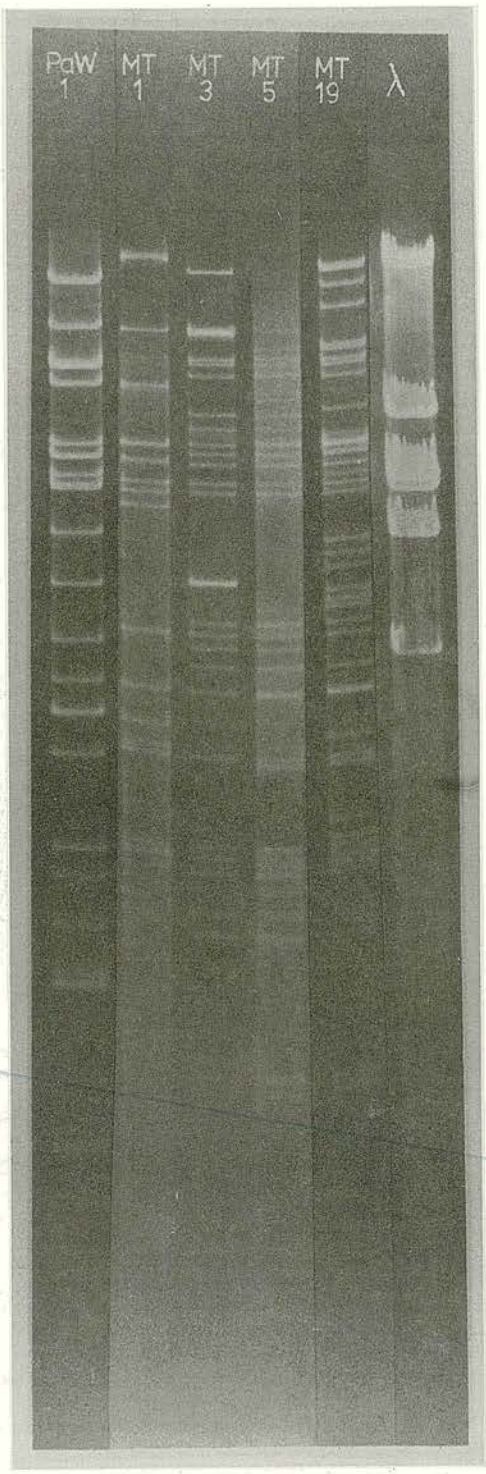

FIG. 2. *Agarose gels of EcoRI digests of unique species of plasmid DNA from Tol⁺ strains, together with those from λ and PaW1 plasmid DNA.*

by A. Chakrabarty (personal communication). This lower frequency was most probably due to the absence of conjugation after transformation under our conditions. All transformants tested were Tol⁺, conjugally proficient, and auxotrophic for adenine. The Tol⁺ progeny obtained from three independent transformants contained plasmid DNA that was indistinguishable after digestion and gel electrophoresis from the plasmid DNA of *P. putida* mt-2 (Fig. 1). Electron microscopy of these plasmid species showed that by this criterion too the plasmid was indistinguishable from that of the parental strain. Simultaneously, attempts were made to prepare plasmid DNA from strain AC34, the recipient strain in these experiments. No plasmid band was detectable in ethidium bromide-CsCl density gradients after several attempted isolations from this strain. Transformation experiments with plasmid DNA from strain MT17 gave similar results. These plasmid species can therefore be referred to unequivocally as TOL plasmids.

With plasmid species other than those of the *P. putida* mt-2 type, we have not yet been able to get transformation. However, in those cases where only one plasmid species is present, we can assign the TOL function to those plasmids on genetic criteria. Moreover, strains MT3-B1, MT15-B1, MT16-B1, MT19-B1, MT20-B1, and MT21-B1, which are Tol⁻ segregants (17), were found to be plasmid-free. The question of whether both plasmids in strains MT1 and MT3 are involved in the TOL-specified function is still open. The significance of the heterogeneity of plasmid population in strain MT14, and of the molecular basis of the TOL-specified function in strain MT20, will be the subjects of further reports.

DISCUSSION

We have shown that in at least some of the soil pseudomonads we have studied, the TOL function is plasmid borne. It is striking that in a number of independently isolated strains (*P. putida* mt-2, MT16, MT17, MT18, and MT21), the TOL plasmids must be very closely related. This group could also be distinguished from the other Tol⁺ strains studied here by genetic and physiological criteria (17). These results indicate strongly that some plasmids may be very widely distributed in nature. Other plasmids are superficially different, although some molecular relationships may be revealed by annealing or heteroduplex experiments.

It is worth noting that the original TOL⁺ strain, *P. putida* (*arvilla*) mt-2, was apparently isolated in Japan (10), whereas the others

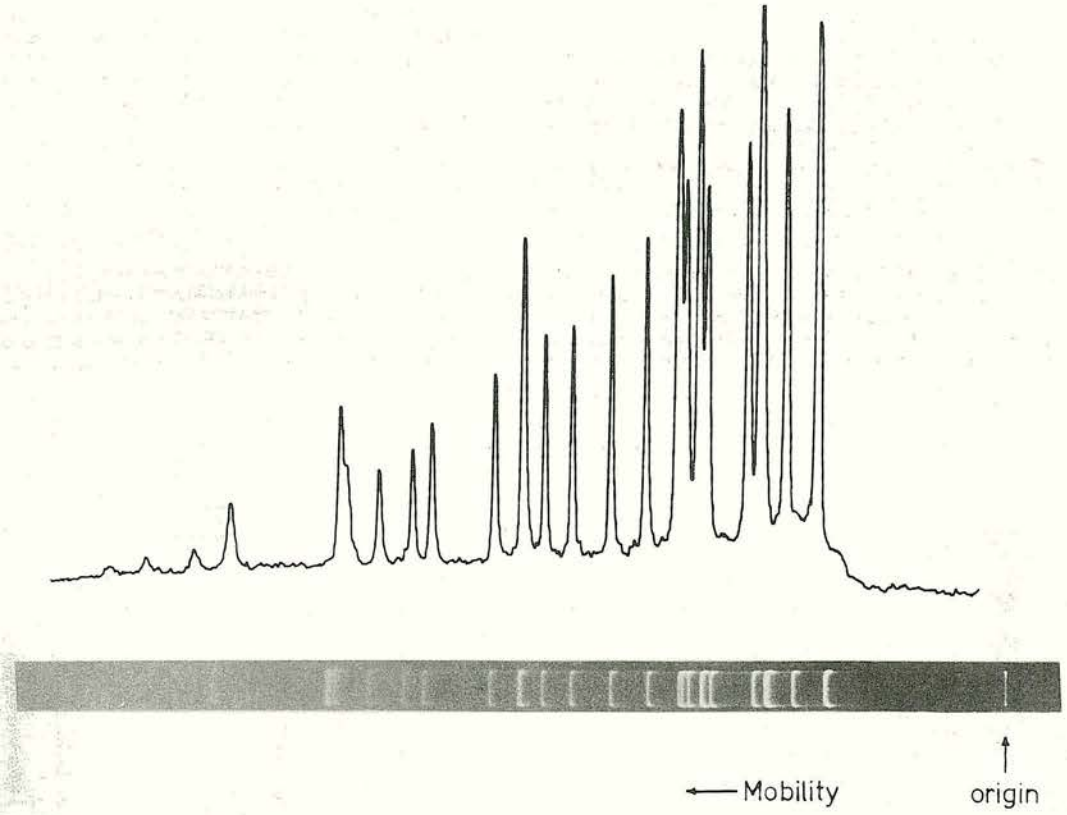

FIG. 3. *Densitometry trace of endonuclease-derived fragments of plasmid pWW0.*

of the series were isolated in Wales. The analogy with the R factors R1, R6, and R100 is striking. These R factors were originally isolated from hospital isolates in England, Germany, and Japan, respectively, but are clearly related (12). Other examples are beginning to emerge in which a group of related plasmids are widely distributed. However, the ubiquity of particular types of R factors could result from disseminating of their host strains as human commensals. Such an explanation seems unlikely for degradative plasmids of soil bacteria.

ACKNOWLEDGMENTS

S.A.B. is a Medical Research Council Scholar. Research in Bangor was supported by the Science Research Council.

We are grateful to A. M. Chakrabarty for suggesting the use of the alkaline denaturation step in the isolation of plasmid DNA.

LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages, p. 445–447. Interscience Publishers Inc., New York.
2. **Alfaro, G., and N. Willetts.** 1972. The relationship between the transfer systems of some bacterial plasmids. Genet. Res. 20:279–289.
3. **Bauchop, T., and S. R. Elsden.** 1960. The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. 23:457–469.
4. **Bazaral, M., and D. R. Helinski.** 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from *Escherichia coli*. J. Mol. Biol. 36:185–194.
5. **Bennett, P. M., and M. H. Richmond.** 1976. Translocation of a discrete piece of deoxyribonucleic acid carrying an *amp* gene between replicons in *Escherichia coli*. J. Bacteriol. 126:1–6.
6. **Chakrabarty, A. M., J. R. Mylroie, D. A. Friello, and J. G. Vacca.** 1975. Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid-linked drug-resistance factor DNA. Proc. Natl. Acad. Sci. U.S.A. 72:3647–3651.
7. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110:667–676.
8. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21:413–428.
9. **Kaiser, A. D., and D. S. Hogness.** 1960. The transformation of *Escherichia coli* with deoxyribonucleic acid isolated from bacteriophage λ *dg*. J. Mol. Biol. 2:392–415.
10. **Nozaki, M., H. Kagiyama, and O. Hayaishi.** 1963. Crystallisation and some properties of metapyrocatechase. Biochem. Biophys. Res. Commun. 11:65–69.
11. **Palchaudhuri, S., and A. Chakrabarty.** 1976. Isolation

of plasmid deoxyribonucleic acid from *Pseudomonas putida*. J. Bacteriol. **126**:410–416.

12. **Sharp, P. A., S. N. Cohen, and N. Davidson.** 1973. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) factors and F factors. J. Mol. Biol. **75**:235–255.
13. **Sharp, P. A., M-T. Hsu, E. Ohtsubo, and N. Davidson.** 1972. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. I. Structure of F-prime factors. J. Mol. Biol. **71**:471–497.
14. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A. **44**:1072–1078.
15. **Thompson, R., S. G. Hughes, and P. Broda.** 1974. Plasmid identification using specific endonucleases. Mol. Gen. Genet. **133**:141–149.
16. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida (arvilla)* mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120**:416–423.
17. **Williams, P. A., and M. J. Worsey.** 1976. Ubiquity of plasmids coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bacteriol. **125**:818–828.
18. **Wong, C. L., and N. W. Dunn.** 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. **23**:227–232.
19. **Worsey, M. J., and P. A. Williams.** 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124**:7–13.

Molec. gen. Genet. 154, 203–204 (1977)

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Two Modes of Loss of the Tol Function from *Pseudomonas putida* mt-2

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Summary. Some of a set of independently arising Tol$^-$ (non toluate-utilising) derivatives of *Pseudomonas putida* mt-2 have lost the unique plasmid present in the parent strain. In others this plasmid has suffered a deletion of a specific region of about 27 Md.

One of the criteria by which the degradation of *m*- and *p*-toluate by *Pseudomonas putida* (*arvilla*) mt-2 was originally judged to be plasmid-specified was the loss of the function at efficiencies higher than those normally found for mutations. In cells carrying the TOL plasmid, degradation of the toluates and benzoate proceeds *via meta* cleavage of catechol. In Tol$^-$ clones, catechol is cleaved and metabolised through the (chromosomally-encoded) *ortho* pathway. A convenient experimental fact is that cells utilising the *ortho* pathway grow faster than those utilising the *meta* pathway. Although both pathways are present in Tol$^+$ strains the *meta* pathway predominates in the dissimilation of catechol since it is substrate-induced in contrast to the product-induced *ortho* pathway. Therefore Tol$^-$ clones overgrow Tol$^+$ clones in growth on benzoate, and can readily be isolated [2].

In the course of characterising plasmids from *P.putida* mt-2 and a number of independently isolated toluate-utilising *Pseudomonas* strains [1, 3], we have examined a number of independently derived Tol$^-$ derivatives of PaWl, and also of strains MT16, MT17, MT18 and MT21, which contain very closely related plasmids. As expected, no plasmid DNA was detectable in some of these strains (MT16Bl and MT21Bl) (Table 1). However, in other cases, for instance strain PaW8, a previously described "cured" derivative of *P.putida* mt-2 derived by mitomycin C treatment [2], plasmid DNA was still present. Electron microscopy of plasmid DNA from strain PaW8 and a benzoate "cured" Tol$^-$ derivative of *P.putida* mt-2, PaW80, gave sizes of 48.6 Md and 48.0 Md respectively. Since the parent plasmid had a size of 78 Md [1], the loss of the Tol function may have occurred by excision of a specific region of the TOL plasmid.

To test this hypothesis, we isolated ten independently arising Tol$^-$ clones from *P.putida* mt-2, after growth on benzoate. Of these, six contained plasmid DNA (Table 1). Such plasmid DNA and plasmid DNA from strains PaW8, PaW80, MT17B1 and MT18B1 was submitted to electrophoresis on agarose gels after digestion with the endonuclease EcoRI (1). The patterns were indistinguishable, so that at this level of resolution the plasmids had all lost the same region(s) (Fig. 1). Comparison with the pattern given by the parental TOL plasmid show that nine fragments have been lost (Fig. 2). No new bands are

Table 1. Independent Tol$^-$ derivatives of strains containing TOL plasmids

Strain	Parent	Mode of curing	Plasmid	Plasmid sizes with standard errors
PaWl	–	–	pWW0	78.1 ± 1.1
PaW8	PaWl	mitomycin C	pWW0-1	48.6 ± 1.0
MT16B1	MT16	benzoate	no plasmid	–
MT17B1	MT17	benzoate	pWW17-1	ND
MT18B1	MT18	benzoate	pWW18-1	ND
MT21B1	MT21	benzoate	no plasmid	–
PaW80	PaW1	benzoate	pWW0-2	48.0 ± 1.1
PaW81, 83, 84, 87, 88, 89	PaW1	benzoate	pWW0-81, 83 84, 87, 88, 89	ND
PaW82, 85, 86, 90	PaW1	benzoate	no plasmid	–

ND = no data

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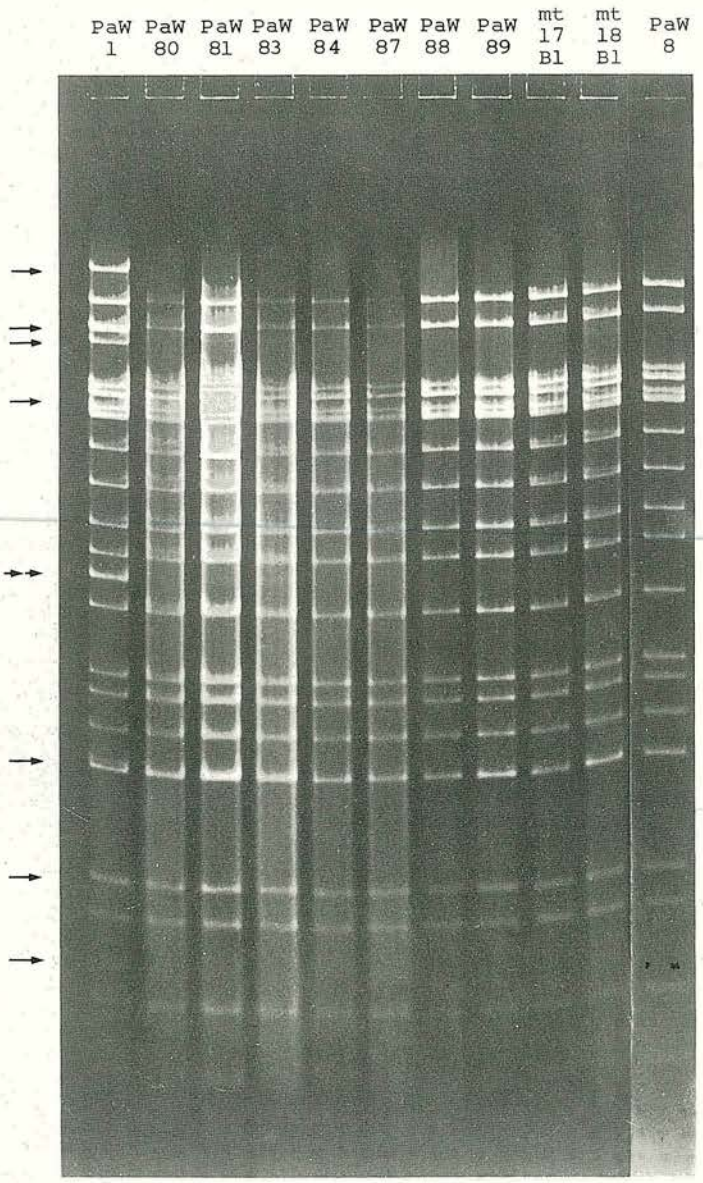

Fig. 1. Agarose gel electrophoresis of fragments of plasmid DNA generated by EcoRI digestion (see Text). The methods used have been described previously

observed; the apparent absence of new bands suggests that the deletion is of a single segment with ends less than about 0.2 Md from EcoRI cleavage sites. An alternative explanation is that new fragments were generated with mobilities that happen to be coincident with those of fragments lost from the parent plasmid.

The molecular weights of the nine fragments, derived from a curve relating mobility to molecular weight using the fragments of known size derived from phage λ, added up to 27 Md. The calculated loss of DNA corresponds adequately with that inferred from electron microscopy of two representative shortened plasmids (see above). We conclude that there are "hotspots" for the excision of a segment that codes for at least part of the TOL pathway. We are currently investigating the molecular basis of this phenomenon.

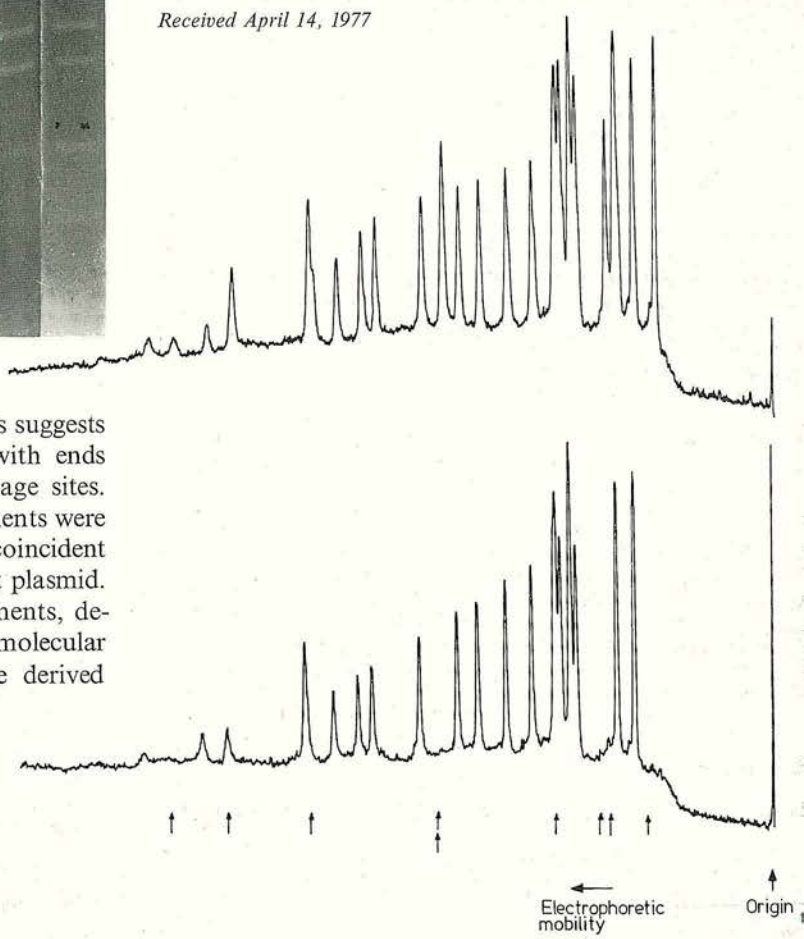

Fig. 2. Comparison of densitometry traces of electrophoresis patterns of EcoRI digested plasmid DNA from strains PaWl (upper trace) and PaW8 (lower trace). The arrows indicate the positions of the nine fragments present in the digest of plasmid pWW0 but not of plasmid pWW0-1

Acknowledgements. S.A.B. is a Medical Research Council scholar. Research in Bangor was supported by the Science Research Council.

References

1. Duggleby, C.J., Bayley, S.A., Worsey, M.J., Williams, P.A., Broda, P.: Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bact. (in press) (1977)
2. Williams, P.A., Murray, K.: Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bact. **120**, 416–423 (1974)
3. Williams, P.A., Worsey, M.J.: Ubiquity of plasmids coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. J. Bact. **125**, 818–828 (1976)

Communicated by B.A. Bridges

Received April 14, 1977

Mol Gen Genet (1981) 184:97–101

MGG

Excision of the 40kb Segment of the TOL Plasmid from *Pseudomonas putida* mt-2 Involves Direct Repeats

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Summary. The TOL plasmid pWWO gives rise to the Tol$^-$ plasmid pWWO-8 due to a specific excision event involving 40 kb of TOL DNA. This event occurs by means of reciprocal recombination involving a pair of directly repeated 1.4 kb sequences. This 40 kb segment is not involved as such in the formation in vivo of hybrid plasmids between TOL and resistance plasmids, notably RP4.

Introduction

The degradation of toluene and *m* and *p*-xylenes by *Pseudomonas putida* mt-2 is due to an inducible enzyme pathway specified by the TOL plasmid pWWO (Williams and Murray 1974; Worsey and Williams 1975). Strains lacking this degradative function can be selected after growth on benzoate, an intermediate in both the plasmid-encoded *meta* pathway and the chromosomally-encoded *ortho* pathway (Williams and Murray 1974). Such loss occurs either by loss of the whole plasmid or by specific excision of a 40 kb segment resulting in the formation of a Tol$^-$ plasmid, for which the first example was pWWO-8 (Bayley et al. 1977). Restriction mapping indicates that the excised region is a contiguous segment of the TOL plasmid pWWO (Downing and Broda 1979).

One possible mechanism for specific excision events is reciprocal recombination between two DNA sequences present in direct repeat at the ends of the excised region (the Campbell model), as in the formation of F$^+$ strains from Hfr strains. In this paper we show results which suggest that this mechanism applies to the formation of the plasmid pWWO-8 from pWWO.

The existence of a Tol transposon has been alluded to by other laboratories after the isolation in vivo of hybrids of TOL with RP4, R91 or R702 (Jacoby et al. 1978; Nakazawa et al. 1977; Chakrabarty et al. 1978; Franklin and Williams 1980; White and Dunn 1977). Our results indicate that the 40 kb segment is not a Tol transposon because the TOL-derived sequences present in the RP4-TOL hybrid plasmids studied are all larger than 40 kb and extend beyond the excised segment at both ends.

Materials and Methods

Bacteria. Bacterial strains and plasmids are shown in Table 1.

Antibiotics were used at the following concentrations: For *Pseudomonas* spp.: Carbenicillin 2 mg/ml; Tetracycline 50 μg/ml; Kanamycin 50 μg/ml. For *E. coli:* Ampicillin 100 μg/ml; Tetracycline 20 μg/ml; Kanamycin 50 μg/ml.

Isolation of Plasmid DNA. For pWWO, pWWO–8, RP4 and all RP4–TOL plasmids a scaled up version of the method of Hansen and Olsen (1978) was used starting with 1 litre cultures.

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Table 1

Strain	Plasmid	Phenotype conferred by plasmid[b]	Size (kb)[c]	Reference
Pseudomonas aeruginosa				
PU21	pED3304	Tol$^+$ Cb^R Kn^R	120	Jacoby et al. 1978
Pseudomonas putida				
PAW1	pWWO	Tol$^+$	117	Williams and Murray 1974
PAW8	pWWO–8	Tol$^-$	77	Bayley et al. 1977
PAW153	pED3300[a]	Tol$^+$ Cb^R Kn^R	120	Franklin and Williams 1980
PAW339	pED3301[a]	Tol$^+$ Cb^R Kn^R	120	Williams (unpublished)
AC810	pED3302[a]	Tol$^+$ Cb^R Kn^R	120	Chakrabarty 1978
AC836	pED3303[a]	Tol$^+$ Cb^R Kn^R Tet^R	115	Chakrabarty (unpublished)
TN2002	pTN2	Tol$^+$ Cb^R Kn^R Tet^R	110	Nakazawa et al. 1978
ED3305	pED3305	Tol$^-$ Cb^R Kn^R	80	This study
Escherichia coli				
ED8654	None	–	–	Borck et al. 1976
ED8654/ pBR322	pBR322	Ap^R Tet^R	4.4	Bolivar et al. 1977
ED3306	pED3306	Ap^R	14.4	This study
ED3307	pED3307	Ap^R	11.6	This study
ED3308	pED3308	Ap^R	11.7	This study

[a] Plasmids from elsewhere that have not previously been assigned proper names
[b] Abbreviations are as follows: Tol$^+$: ability to use *m*-toluate as sole carbon source; Ap: ampicillin; Cb: carbenicillin; Kn: Kanamycin; Tet: tetracycline
[c] Size determined by summation of restriction fragment sizes obtained from relative mobilities on agarose gels

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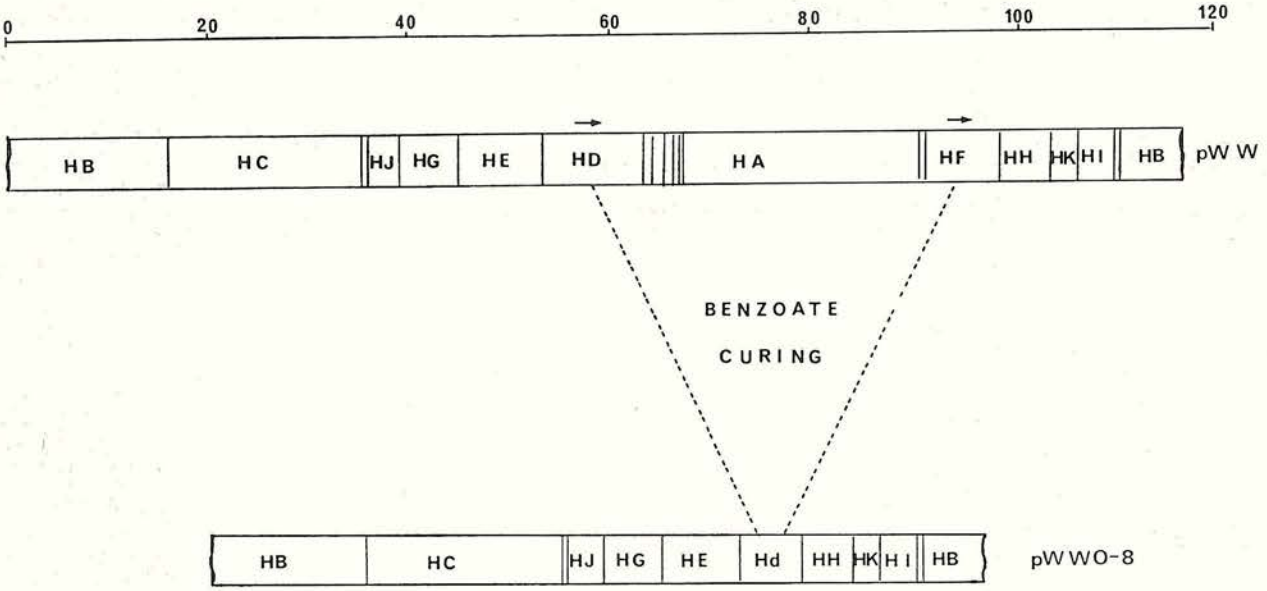


Fig. 1. Benzoate curing of the TOL plasmid pWWO results in the selection of plasmids which have lost the 40 kb segment. The cleavage map for *Hin*dIII shows the ends of the excised region the loss of which resulted in the formation of pWWO–8

pED3306, pED3307, pED3308 and pBR322 were prepared using the cleared lysate procedure of Guerry et al. 1973. The method of Birnboim and Doly (1979) was used for plasmid screening.

Restriction and Ligation of DNA. 1 μg samples of DNA were treated with restriction endonucleases in final volumes of 40 μl in the following buffers all at pH 7.4:

*Bgl*II and *Pst*I	20 mM Tris-HCL, 10 mM $MgCl_2$
*Eco*RI	100 mM Tris-HCL, 10 mM $MgCl_2$, 50 mM NaCl, 2 mM β mercaptoethanol
*Hin*dIII	20 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl
*Hpa*I	20 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM KCl
*Kpn*I	6 mM Tris-HCl, 6 mM $MgCl_2$, 6 mM NaCl, 6 mM β mercaptoethanol
*Xho*I	8 mM Tris HCl, 10 mM $MgCl_2$, 150 mM NaCl, 6 mM β mercaptoethanol.

Incubations for 90 min at 37° C included 1 unit of enzyme. Digestion was terminated by incubating at 70° C for 10 min.

For the ligation of *Hin*dIII cohesive ends, 1 μg of restricted pBR322 DNA treated with alkaline phosphatase and 2 μg of restricted pWWO or pWWO-8 DNA were ligated in a final volume of 60 μl buffer containing 6 mM Tris-HCl (pH 7.4), 1 mM EDTA 10 mM MgCl, 10 mM β mercaptoethanol and 0.1 mM ATP, by incubating, after inactivating the restriction enzyme, with 2 units of T4 ligase at 10° C overnight.

*Eco*RI, *Bgl*II, *Xho*I and *Kpn*I were bought from Bethesda Research Laboratories. *Hpa*I was a gift from Mr. I. Garner. *Pst*I was a gift from Mr. A.M.C. Brown. *Hin*dIII and T4 ligase were gifts from Mrs. S. Bruce.

Transformation. ED8654 was transformed with the ligated mixture by the method of Humphreys et al. 1979. Transformed cells were plated out onto nutrient agar containing 100 μg/ml ampicillin. Individual colonies were then tested for tetracycline sensitivity as any fragment cloned into the *Hin*dIII site of pBR322 would inactivate the tetracycline resistance determinant. Putative clones were screened for the size of the plasmids they carried using the Birnboim and Doly (1979) procedure.

Agarose Gel Electrophoresis. Horizontal 0.7% agarose slab gels were run submerged in troughs of borate electrophoresis buffer containing 89 mM boric acid, 89 mM Tris base, 2.5 mM EDTA pH 8.2. After electrophoresis overnight at 50 volts the gels were stained with ethidium bromide and photographed under U.V. light.

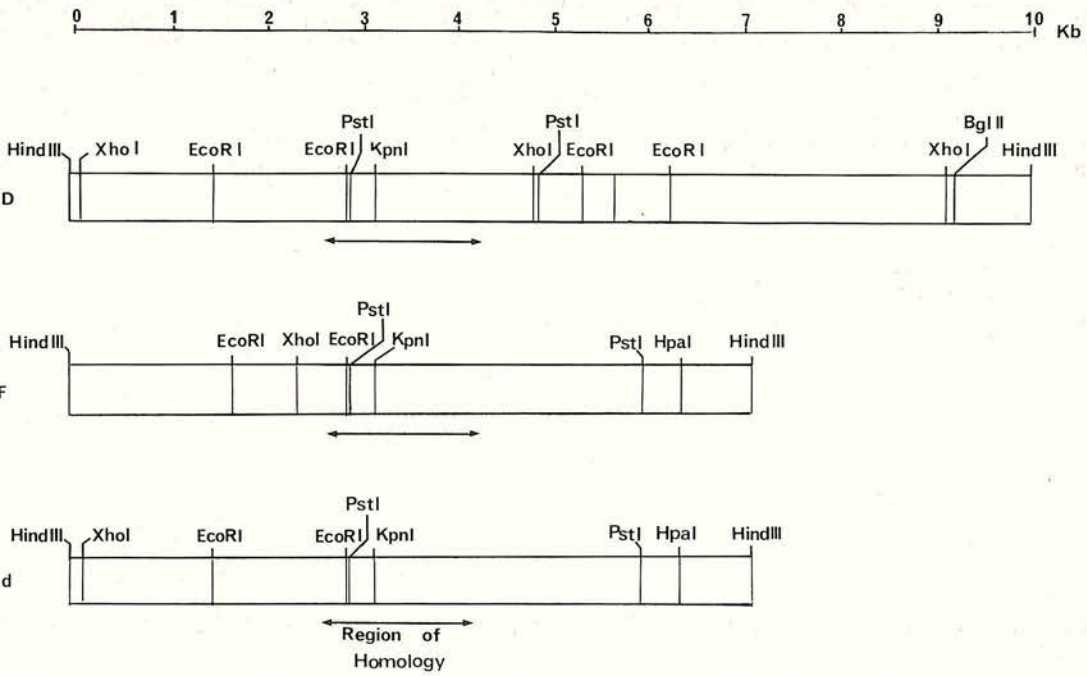
Heteroduplex analysis was carried out using the method of Davis et al. 1971. pED3306 and peED3307 were cut with *Bgl*II and *Hpa*I respectively to linearise the molecules before denaturation.

3. Results and Discussion

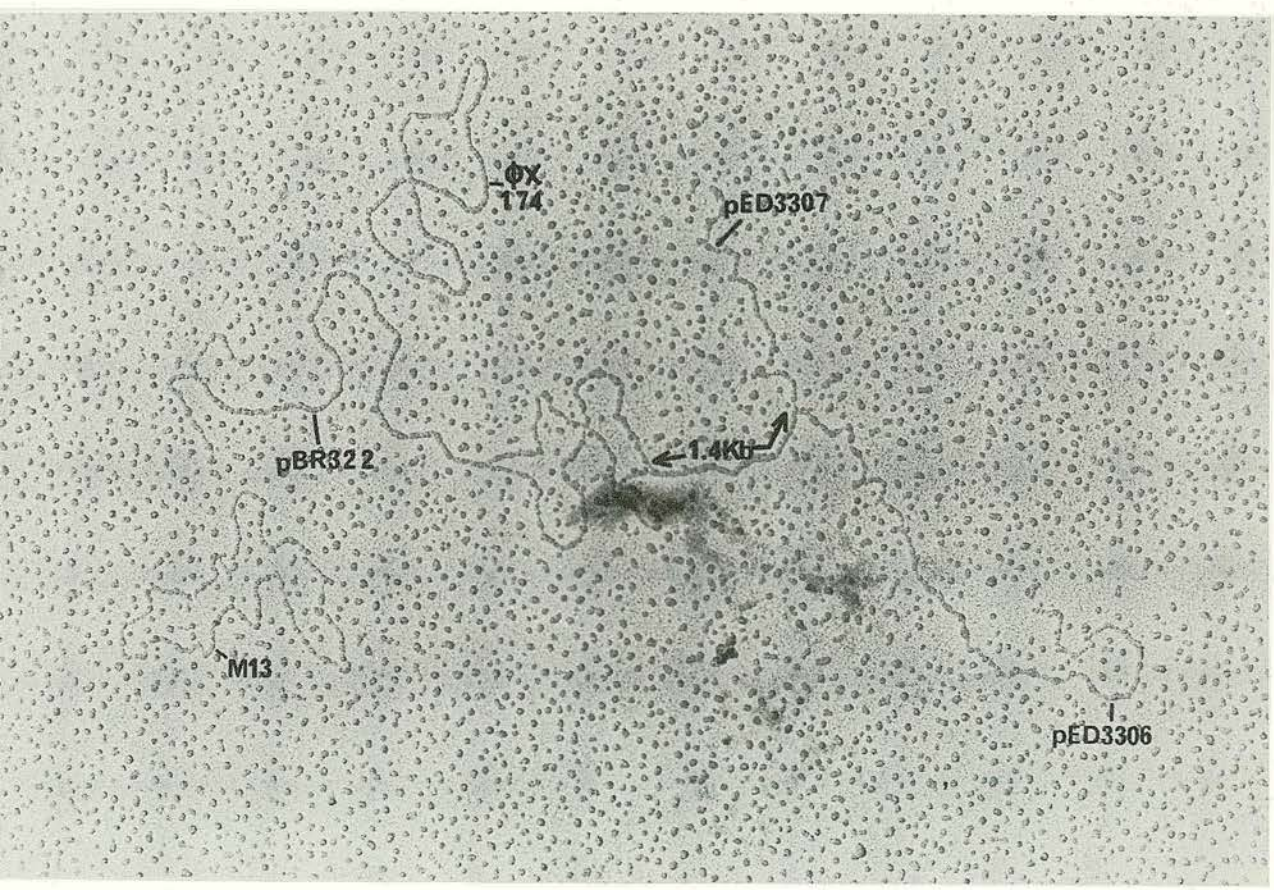
Using our knowledge of the *Hin*dIII restriction map of pWWO and pWWO–8, we deduced that the ends of the specifically excised 40 kb region lay in the *Hin*dIII fragments HD and HF of pWWO (Downing and Broda 1979) (see Fig. 1). In order to obtain more information on the structure of these fragments, they were cloned into the vector plasmid pBR322 (Bolivar 1977) in a shotgun cloning experiment using pWWO DNA. The novel *Hin*dIII fragment Hd in pWWO–8, formed as a consequence of the excision event, was cloned in similar manner using pWWO–8 DNA (see Materials and Methods).

The restriction maps of these three fragments, obtained using the hybrid plasmids pED3306, pED3307 and pED3308 respectively, are shown in Fig. 2. The similarities between these fragments with respect to the distribution of restriction enzyme sites are apparent and suggest that there is a repeated DNA sequence in all three plasmids. Since the orientations of fragments HD and HF in the vector plasmids were obvious from the restriction mapping, and their orientation in pWWO was known from the asymmetric distribution of *Xho*I sites, the predicted pair of repeated sequences would lie in the same orientation in pWWO.

In order to show whether there was in fact such a common sequence in HD and HF, heteroduplex molecules were made using pED3306 and pED3307. An electron micrograph of one



ig. 2. Restriction maps of *Hin*dIII fragments HD and HF from pWWO and of Hd, the novel *Hin*dIII restriction fragment present in pWWO–8, nowing similarities between them



g. 3. Heteroduplex analysis of pED3306 and pED3307 showing the 1.4 kb repeat which is in direct orientation in pWWO. Phage M13 NA was used as a single-stranded standard. Phage ϕX174 was used as double-stranded standard

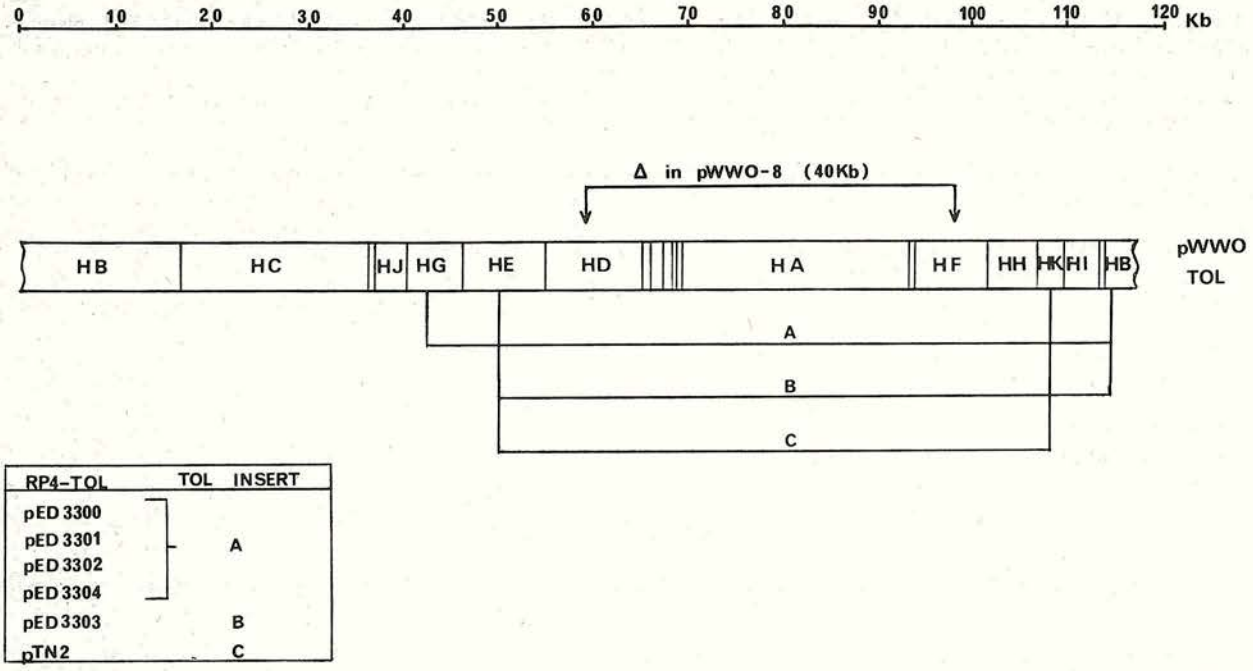


Fig. 4. Amounts of TOL DNA present in different RP4–TOL hybrid plasmids. In the case of pTN2, we confirm data of Nakazawa et al. 1980

such molecule (of twelve measured) is shown in Fig. 3. A repeated region of 1.4 kb was revealed to be approximately 2.8 kb from the end of the pBR322 duplex on the linear molecule. This is consistent with the position of the common restriction enzyme sites and therefore locates and sizes the repeated sequences accurately. The pattern of target sequences on the restriction map of Hd shows that it has only one copy of this repeat; this copy defines the HD/HF boundary in the hybrid fragment. Thus excision of the 40 kb segment is due to reciprocal recombination between direct repeats of 1.4 kb in the DNA sequence of the *Hin*dIII fragments HD and HF of pWWO.

Inspection shows that the distribution of endonuclease cleavage sites on this 1.4 kb segment are different from those reported for IS sequences 2, 3 and 4 (Ghosal and Saedler 1979; Sommer et al. 1979; Haberman et al. 1979). Both the target sites and sizes differ from those in IS1, IS8 and IS21 (Ohtsubo and Ohtsubo 1978; Depicker et al. 1980; Willetts et al. 1981).

It was interesting to speculate whether the 40 kb excised region was also implicated in the formation in vivo of the RP4–TOL hybrid plasmids mentioned earlier. To test this, six independently arising RP4–TOL plasmids were examined to see if the 40 kb segment alone was involved. Plasmid DNA was isolated from all six strains, digested with *Hin*dIII and run out on an agarose gel. In each case the amount of TOL DNA present in these RP4–TOL hybrids is more than 40 kb and extends beyond the excised segment at both ends. We have also demonstrated, e.g. in the formation of pED3305 from pED3301, that the 40 kb segment can still be lost from these plasmids leaving a segment of TOL DNA on RP4. However Fig. 4 shows that the TOL moieties present in the different RP4–TOL plasmids are not identical; this rules out a transposition model involving a unique segment of the TOL plasmid. We are currently investigating the mechanism of formation of these plasmids.

Acknowledgements. We thank Pamela Beattie for the E.M. work, Joanna Rennie for photography and the Science and Engineering Research Council for support. P.M. is a Medical Research Council scholar.

References

Bayley SA, Duggleby C, Worsey MJ, Williams PA, Hardy KG, Broda P (1977) Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol Gen Genet 154:203–204

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acid Res 7:1513–1524

Bolivar F, Rodriguez RL, Greene PJ, Betlach HC, Heynecker HL, Boyer HW, Crosa JH, Falkow S (1977) Construction and characterisation of new cloning vehicles. II. A multiple cloning system. Gene 2:75–93

Borck K, Beggs JD, Brammar WJ, Hopkins AS, Murray NE (1976) The construction in vitro of transducing derivatives of phage lambda. Mol Gen Genet 146:199–207

Campbell A (1962) Episomes. Advances in Genetics 11:101–145

Chakrabarty AM, Friello DA, Bopp LH (1978) Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various organisms. Proc Natl Acad Sci USA 75:3109–3122

Davis RW, Simon M, Davidson N (1971) Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. In: Grossman L and Moldave K (eds) Methods in enzymology, Vol 21. Academic Press In., New York

Depicker A, De Block M, Inzé D, Van Montagu M, Schell J (1980) IS-like element IS8 in RP4 plasmid and its involvement in cointegration. Gene 10:329–338

Downing RG, Duggleby CJ, Villems R, Broda P (1979) An endonuclease cleavage map of the plasmid pWWO–8, a derivative of the TOL plasmid of *Pseudomonas putida* mt-2. Mol Gen Genet 168:97–99

Downing RG, Broda P (1979) A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. Mol Gen Genet 177:189–191

Ghosal D, Sommer H, Saedler H (1979) Nucleotide sequence of the transposable DNA element IS2. Nucl Acid Res 6:1111–1122

Guerry P, Leblanc DJ, Falkow S (1973) General method for isolation of plasmid deoxyribonucleic acid. J Bacteriol 116:1064–1066

Haberman P, Klaer R, Kuhn S, Starlinger P (1979) IS4 is found between eleven and twelve base pair duplications. Mol Gen Genet 175:368–373

Hansen JB, Olsen RH (1978) Isolation of large bacterial plasmids

and characterisation of the P2 incompatibility group plasmids pMG1 and pMG5. J Bacteriol 135:227–238

Humphreys GO, Weston A, Brown MGM, Saunders JR (1979) Plasmid transformation of *Escherichia coli*. In: Glover SW and Butler LO (eds) Transformation 1978. Cotswold Press, Oxford, pp 254–279

Jacoby GA, Rogers JE, Jacob AE, Hedges RW (1978) Transposition of toluene degrading genes and expression in *E. coli*. Nature 274:179–180

Nakazawa T, Hagashi E, Yokota T, Ebina Y, Nakazawa A (1978) Isolation of TOL and RP4 recombinants by integrative suppression. J Bacteriol 134:270–277

Nakazawa T, Inouye S, Nakazawa A (1980) Physical and functional mapping of RP4–TOL plasmid recombinants: analysis of insertion and deletion mutants. J Bacteriol 144:223–231

Ohtsubo H, Ohtsubo E (1978) Nucleotide sequence analysis of an insertion element ISI. Proc Natl Acad Sci USA 75:615–619

Ommer J, Cullum J, Saedler J (1979) Integration of IS3 into IS2 generates a short sequence duplication. Mol Gen Genet 177:85–89

White GP, Dunn NW (1977) Apparent fusion of the TOL plasmid with the R91 drug resistance plasmid in *Pseudomonas aeruginosa*. Aust J Biol Sci 30:345–355

Willetts NS, Crowther C, Holloway BW (1981) The insertion sequence IS21 of R68.45 and the molecular basis for mobilization of the bacterial chromosome. Plasmid 6:30–52

Williams PA, Murray K (1974) Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J Bacteriol 120:416–413

Worsey MJ, Williams PA (1975) Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J Bacteriol 124:7–13

Communicated by E. Bautz

Received August 3, 1981

Mol Gen Genet (1981) 184:102–106

MGG

The Use of Cloned *nif* (Nitrogen Fixation) DNA to Investigate Transcriptional Regulation of *nif* Expression in *Klebsiella pneumoniae*

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Summary. Some restriction endonuclease fragments of *nif* DNA, when carried on small multicopy plasmids, inhibited *nif* expression in *Klebsiella pneumoniae*. A study of this inhibitory effect revealed, (1) that overproduction of the *nif*L gene product inhibited transcription of two *nif* operons examined, *nif*J and *nif*HDKY and, (2) that when transcription was initiated from the promoter of the *nif*HDKY operon on multicopy plasmids there was a corresponding decrease in the transcription rates of the chromosomally located *nif*J and *nif*HDKY but not the *nif*LA operon. Studies of transcription in vivo also showed that the *nif*A gene product was essential for transcription initiation from the *nif*HDKY and *nif*BQ promoters. These results, taken with earlier observations (see Discussion) provide evidence that the *nif*L and *nif*A gene products are respectively a repressor and activator of *nif* transcription initiation from all *nif* promoters except that of the *nif*LA operon.

Introduction

The nitrogen fixation (*nif*) gene cluster of *Klebsiella pneumoniae* is linked to *his* and consists of at least 17 genes arranged in 7 or 8 operons (MacNeil et al. 1978; Riedel et al. 1979; Merrick et al. 1980; Pühler and Klipp 1981). The *nif* genes were originally cloned as several large overlapping fragments in small plasmid vectors (Cannon et al. 1977, 1979; Cannon 1978). Detailed physical mapping of these genes (Riedel et al. 1979; Pühler and Klipp 1981) has facilitated the cloning of separate *nif* genes and their promoters.

Complementation tests using the original clones and *nif* chromosomal mutations were inconclusive because the strains used were *Rec*A$^+$ and it was therefore not possible to distinguish between a low level of complementation and a high rate of recombination. One of the multicopy *nif* plasmids, pSA30, had an inhibitory effect on *nif* expression in a *Nif*$^+$ strain and Cannon et al. (1979) suggested that this could be explained by assuming a common transcriptional activator for *nif* promoters which was preferentially bound to multiple copies of the *nif*HDKY promoter on pSA30 and thus reduced transcriptional activation from the single copies of weaker promoters on the chromosome. Ausubel, Cannon and Riedel (unpublished) found that another *nif* plasmid, pGR112, which carries the *nif* regulatory genes *nif*L and *nif*A, also inhibited *nif* expression in *Nif*$^+$ *Rec*A$^+$ strains.

We have further investigated the inhibitory effect of some *nif* clones on *nif* expression as part of an attempt to unravel the regulatory mechanism of *nif* transcription. Our results show that transcription of some chromosomal *nif* genes is drastically decreased by overproduction of the *nif*L gene product and that transcription from multiple copies of the *nif*HDKY promoter on a small plasmid resulted in a corresponding decrease in *nif* transcription from the chromosome. We have also found that the *nif*A gene product is essential for transcription initiation from the *nif*HDKY and *nif*BQ promoters.

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Materials and Methods

Bacterial Strains and Plasmids. These are listed in Table 1.

Media, Amino-acids and Antibiotics. Complete medium was LB and nitrogen deficient medium was NFDM as described by Cannon et al. (1979). Amino-acids were used at a final concentration of 25 μg/ml. Antibiotics were added at the following final concentrations (μg/ml): – tetracycline, 10; chloramphenicol, 25; carbenicillin, 200; kanamycin, 25.

Plasmid Construction. The plasmids constructed are described in Table 2. They were selected by size after colony screening.

Table 1. Bacterial strains and plasmids

	Genotype or phenotype	Reference or Source
Klebsiella pneumoniae		
UNF928	*his*D2 *hsd*R1 *rec*A56 *sbl*300::Tn10 Tc^S	This paper
UNF714	*his*D2 *hsd*R1 Sm^R *nif*A2263 *rec*A56 *sbl*300::Tn10	M. Merrick
Plasmids		
pBR322	Tc Ap	Bolivar et al. (1977)
pACYC184	Tc Cm	Chang and Cohen (1978)
pACYC177	Km Ap	,,
pBR327	Tc Ap	Soberon et al. (1980)
pBR328	Tc Cm Ap	,,
pSA30	Tc *nif*HDKY	Cannon et al. (1979)
pGR112	Tc *nif*LABQ	Riedel et al. (1979)
pWK27.15-7	pSA30 *nif*H::Tn5	W. Klipp
pWK27.51-10	pSA30 *nif*D::Tn5	,,
pWK27.77-3	pSA30 *nif*K::Tn5	,,
pWK27.26-1	pSA30 *nif*Y::Tn5	,,
pWK23.34	pGR112 *nif*A::Tn5	,,
pWK23.7	pGR112 *nif*L::Tn5	,,
pGR124	pGR112 *nif*B::Tn5	G. Riedel

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RESTRICTION ENDONUCLEASE MAPPING OF DNA USING IN SITU DIGESTION IN TWO-DIMENSIONAL GELS

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Received 9 March 1978

1. Introduction

Physical mapping of DNA with restriction endonucleases is an important preliminary step for many studies on plasmids, viruses and isolated fragments of complex genomes (reviewed [1]). However, the difficulties encountered in such mapping greatly increase with larger molecules.

The approach most used for ascertaining which fragments of an endonuclease digest are adjacent in the intact molecule has been to isolate partial digest products, and to determine the fragments that they contain. The experiments described below show that this approach can be simplified by dispensing with the isolation of the partial digest products. Instead, after electrophoresis of the partial digest, the DNA is digested to completion with the same or another enzyme within the agarose gel, and the products are then resolved by electrophoresis in the second dimension.

2. Experimental

Plasmid pWWO-84 was isolated from *Pseudomonas putida* as in [2,3]. Digestion of phage λcI857 DNA and plasmid DNA with endonucleases *Eco*R1 and/or *Hin*dIII was carried out in 0.01 M Tris–HCl buffer, pH 7.5, containing 0.01 M $MgCl_2$, 0.05 M NaCl and 0.01 M 2-mercaptoethanol, at 37°C for complete cleavage and at 20°C for partial cleavage. The enzymes were inactivated by heating at 70°C for 10 min followed by cooling on ice. Samples were concentrated in a vacuum desiccator and mixed with loading buffer as in [2].

Electrophoresis in the first dimension was carried out in 0.7% agarose rod gels, (3 × 200 mm) in 0.036 M Tris, 0.03 M NaH_2PO_4 and 0.001 M EDTA, pH 7.7 [4], at constant voltage (80 V) until the bromophenol blue marker had migrated 12 cm.

Digestion of DNA within the gel was carried out after the piece of rod gel containing the DNA was excised and dialysed against enzyme buffer for 90 min. The gel was then placed in a tube of internal diameter 1 mm greater than the diameter of the rod, incubated for 10 h at 37°C in buffer containing 10 units *Eco*R1/0.1 ml and afterwards dialysed against the electrophoresis buffer of the first dimension. For electrophoresis in the second dimension, the rod gel was placed between two glass plates (200 mm square) held apart by 3 mm perspex strips at the sides. It was then overlayered with a 2 mm layer of the first dimension agarose gel such that the third (bottom) edge of the gel-casting block was sealed. When set, the whole was filled with either 1% or 1.5% agarose gel in 0.085 M Tris–borate buffer, pH 8.3 containing 0.025 M EDTA [5]. This combination of buffer systems has been used in [6]. Either upward or downward vertical electrophoresis of such gels was carried out at constant voltage (150 V). Horizontal electrophoresis of gels that had been set onto a single glass plate, using buffer-saturated wicks of J-cloth (Jeyes Group Ltd, Thetford, Norfolk) laid upon the surface of the gel, was also performed in several cases.

Definition: 1 unit of endonuclease is the amount of enzyme which cleaves 1 μg of λ DNA in 1 h at 37°C.

3. Results and discussion

The utility of this approach depends upon the restriction endonuclease being able to enter the agarose gel and to cleave the DNA within it. To develop this method we used phage λ DNA and *Eco*R1 endonuclease, since the cleavage sites have been well characterised [7].

Electrophoresis of intact phage λ DNA was carried out in rod gels and the section containing DNA (located by staining a rod gel run in parallel) was digested with endonuclease *Eco*R1 (see section 2). Good resolution of the six known digest products in the second dimension (fig.1) shows that complete digestion of λ DNA within the gel is achieved under these conditions, and that there is insignificant diffusion of DNA within or out of the gel rod during the various manipulations. In further experiments we found that most DNA was cleaved to completion within 2 h. Although small amounts of uncleaved DNA remained after 8 h digestion, for most purposes an incubation of from 4–6 h should suffice. Under similar conditions, endonuclease *Hin*dIII also cleaves λ DNA within the gel.

It is obvious that when partially digested DNA is separated by electrophoresis and then redigested within the gel, more complex patterns may result. Since the conditions of electrophoresis in both dimensions are similar, those fragments unaffected by the second digestion will lie on the diagonal. In contrast, two or more fragments formed during the second digestion will move relatively further in the second dimension than did their parent molecule in the first. Such fragments will then lie in a vertical line below the diagonal. The knowledge of which fragments came from a particular partial digest product can then be used as the basis for map construction. Two-dimensional electrophoretograms of the complete and partial digests of λ DNA with endonuclease *Eco*R1 are shown in fig.2a and fig.2b respectively. The singlet fragments (lettered according to their size) lie on the diagonal, whereas those arising from in situ digestion lie below it (fig.2b). By comparing their positions with those of the products of complete initial digestion it can be inferred that the partial digest contained the following complex fragments: EDB(a), DB(b), DE(c) and CF(d). Close examination also revealed a partial digest product AEDB and BCF. The composition of those fragments allows us to arrive unambiguously at the order for the six fragments first determined [7] (fig.3).

In a case where the pattern of endonuclease *Eco*R1 fragments was too complex for direct two-dimensional analysis, a different approach was used. A derivative of the *Pseudomonas putida* degradative plasmid TOL,

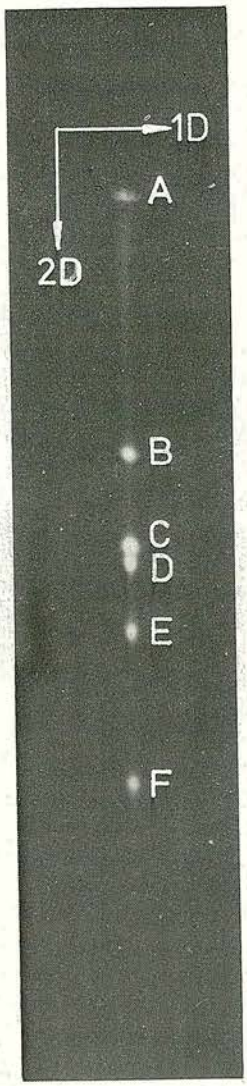

Fig.1. Digestion of λ DNA within an agarose gel. 0.3 μg λ DNA was electrophoresed in an 0.7% agarose rod gel. The section containing DNA was cut out from the gel rod, dialysed, digested with endonuclease *Eco*R1 and set within the second-dimension agarose gell (1%) as described in the text.

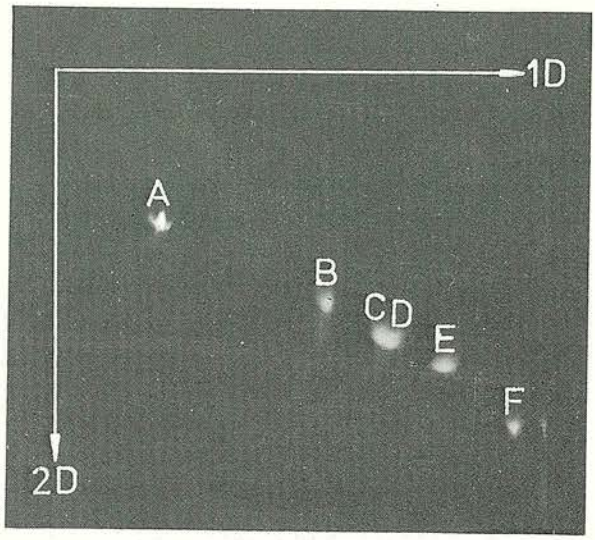

Fig.2a

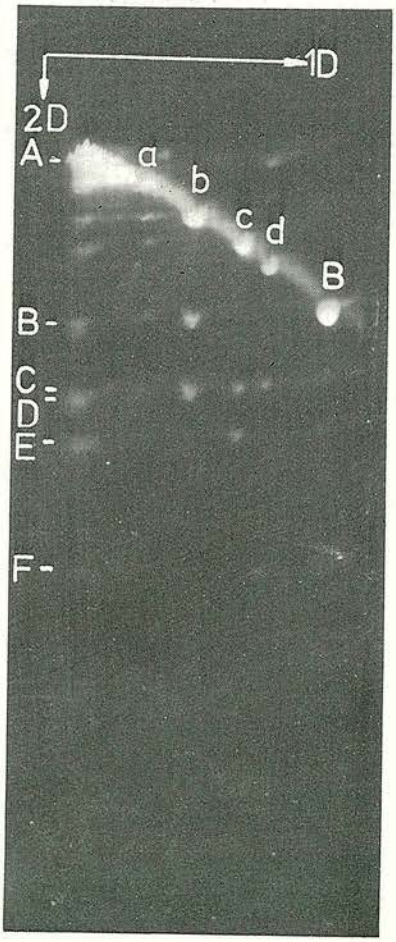

Fig.2b

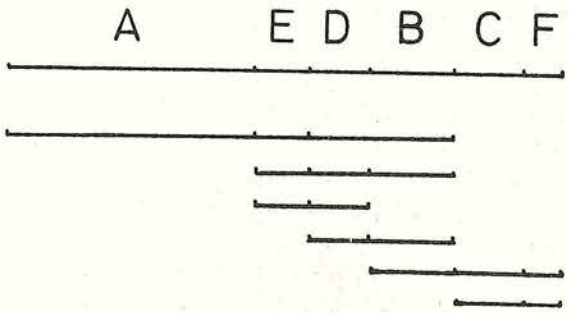

Fig.3. Schematic representation of λcI857 DNA restriction map with endonuclease *Eco*R1 [7] together with a map of λ DNA partial digest products as deduced from two-dimensional gel electrophoresis data.

pWWO-84 [3], was first completely digested with endonuclease *Hin*dIII, giving a comparatively simple set of fragments, which were then redigested by endonuclease *Eco*R1 within the gel. Two *Hin*dIII fragments, F and H, do not have *Eco*R1 sites, whereas the other fragments each give a characteristic pattern of secondary products (fig.4b). We are currently using this approach to map pWWO-84 and the parental TOL plasmid.

An extension of this technique could be used to establish whether different sized plasmids, harboured by a single bacterial strain contain similar sequences of DNA. Here, resolution of the intact plasmids in the first dimension would be followed by in situ digestion and comparison of the fragmentation patterns given by the different size species in the second dimension.

Fig.2a. Two-dimensional diagonal gel electrophoresis of a complete endonuclease *Eco*R1 digest of λ DNA using 1 μg DNA. Electrophoresis was carried out as described in the text.
Fig.2b. Two-dimensional diagonal gel electrophoresis of a partial endonuclease *Eco*R1 digest of λ DNA in the first dimension followed by complete digestion within the gel before the second dimension, as described in the text. (a–d) various partial digest products not digested to completion during the secondary digestion. Only a part of the gel containing partial digest products is represented.

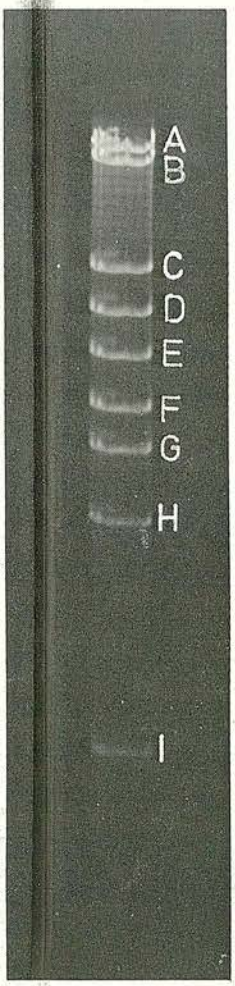

Fig.4a

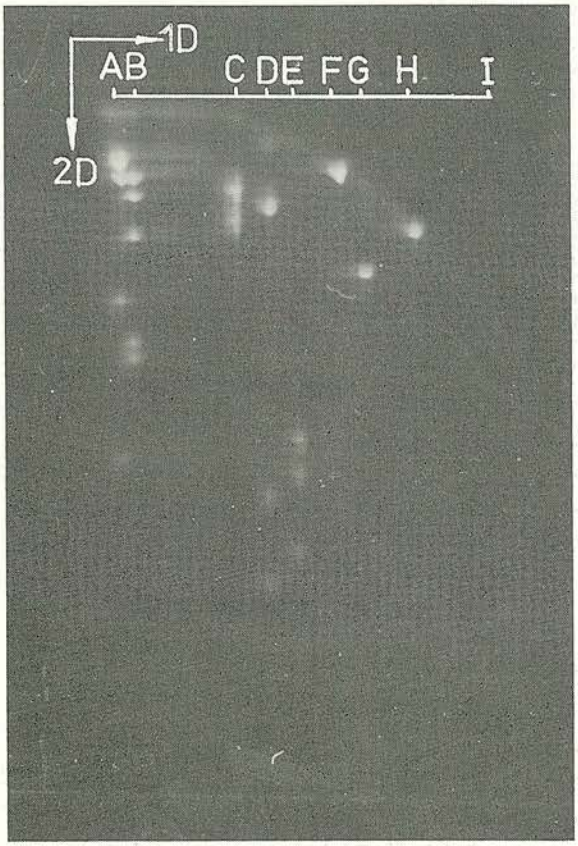

Fig.4b

Fig.4a. Separation of *Hin*dIII fragments from pWWO-84 on 1% agarose gel. 1 μg of plasmid DNA was digested with 2 units of *Hin*dIII at 37°C for 2 h.
Fig.4b. Diagonal gel electrophoresis of pWWO-84 DNA. A complete digest with *Hin*dIII was run in the first dimension; *Eco*R1 digestion in situ was followed by electrophoresis in the second dimension.

Acknowledgements

R. V. is a visiting British Council Scholar from Tartu University, Estonia, USSR.

References

[1] Roberts, R. J. (1976) CRC Crit. Rev. Biochem. 4, 123–164.
[2] Duggleby, C. J., Bayley, A. S., Worsey, M. J., Williams, P. A. and Broda, P. (1977) J. Bacteriol. 130, 1274–1280.
[3] Bayley, S. A., Duggleby, C. J., Worsey, M. J., Williams, P. A., Hardy, K. G. and Broda, P. (1977) Molec. Gen. Genet. 154, 203–204.
[4] Hayward, G. S. (1972) Virology 49, 342–344.
[5] Peacock, A. C. and Dingman, C. W. (1967) Biochemistry 6, 1818–1827.
[6] Derynck, P. and Fiers, W. (1977) J. Mol. Biol. 110, 387–404.
[7] Thomas, M. and Davies, R. W. (1975) J. Mol. Biol. 91, 315–328.

Molec. gen. Genet. 168, 97–99 (1979)

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An Endonuclease Cleavage Map of the Plasmid pWWO-8, a Derivative of the TOL Plasmid of *Pseudomonas putida* mt-2

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Summary. Cleavage sites on the pWWO-8 plasmid were determined for the restriction endonucleases HindIII and XhoI. Terminal labelling using DNA polymerase I was particularly useful both for the characterisation of the smaller cleavage products and for confirmation of the order of fragments in the intact plasmid.

Introduction

The degradation of toluene and the xylenes by *P. putida* mt-2 is mediated by enzymes specified by the TOL plasmid pWWO, which has a size of about 118 kb (Duggleby et al., 1977). The loss of this degradative function, for instance in the formation of plasmid pWWO-8, has been correlated with the loss of about 40 kb of this DNA (Bayley et al., 1977). As a first step in the structural analysis of pWWO, we have constructed an endonuclease cleavage map of the pWWO-8 plasmid using two enzymes, HindIII and XhoI.

Because of its size, pWWO-8 yields a moderate number of fragments with a wide range of sizes, with most enzymes tested. This dictated the choice of enzymes used. Our analysis depended primarily on the accurate determination of the molecular weight sizes of products of digestion with one, the other, or both enzymes. This was done using a calibration curve relating mobility on agarose gels with the previously-determined sizes of a number of λ DNA restriction fragments. In addition, partial digestion products were used to confirm various assignments, and in some cases to distinguish between alternative orders. Further, DNA polymerase I was used as an end-labelling reagent in the characterisation of the composition of some cleavage products; this was possible since the endonuclease-generated fragments have single-stranded ends. The particular value of such terminal-labelling is that subsequent autoradiography reveals *all* fragments, irrespective of size, equally effectively. It is therefore especially useful in the analysis of the smaller digestion products.

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Materials and Methods

Plasmid DNA was isolated as described by Duggleby et al. (1977), or according to the method of Hansen and Olsen (1978).

Endonuclease HindIII was obtained from Miles. XhoI was prepared by J. Atkins. For both enzymes, plasmid DNA was digested at 37° C in 10 mM Tris-HCl buffer, pH 7.5, which included 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 50 mM NaCl.

Agarose gel electrophoresis was performed with horizontal gels in Tris-acetate buffer (pH 8.2). The following size markers were used: intact linear phage λ DNA; λ DNA digested with BamHI, EcoRI, HindIII, KpnI, SalI, and also EcoRI and HindIII together; plasmid pBR322 digested with HpaII.

Terminal-labelling of restriction fragments was carried out using $|\alpha\text{-}^{32}P|$ dGTP (Radiochemicals Centre, Amersham) and Klenow DNA polymerase I (Boehringer, Mannheim). Approximately 0.5 µg DNA were placed in a vial containing 1 µl of 1.0 mCi/ml $|\alpha\text{-}^{32}P|$ dGTP (sp. act. 350 Ci/mM) (dry); in addition the reaction mixture contained 1 µl of a freshly made solution of dTTP, dCTP and dATP (all at 0.12 mM) in 6 mM Tris-HCl pH 7.4; 6 mM $MgCl_2$; 50 mM NaCl; 1 mM dithiothreitol, and 0.2 µ Klenow enzyme. After incubation at room temperature for 30 min., the enzyme was inactivated by heating at 75° C for 20 min. In the case of double digests, terminal-labelling was carried out either after digestion by one or both enzymes. Following electrophoresis, the agarose gels were dried in a slab dryer (Bio-Rad Laboratories) and autoradiography was carried out overnight.

Results and Discussion

Digestion of the plasmid by a number of enzymes was tested to establish which combination was most appropriate for use in mapping. The approximate number of easily visible bands (that is, bands containing one or more fragments with sizes greater than 0.5 kb) were as follows: BamHI: 15; BclI: 23; HindII: > 50; HindIII: 9; PstI: 24; SmaI: 11; EcoRI: 19; XhoI: 3.

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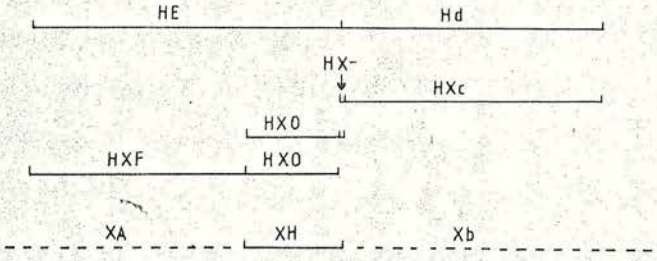

Fig. 1. The orientation of fragments HE and Hd relative to XhoI fragments

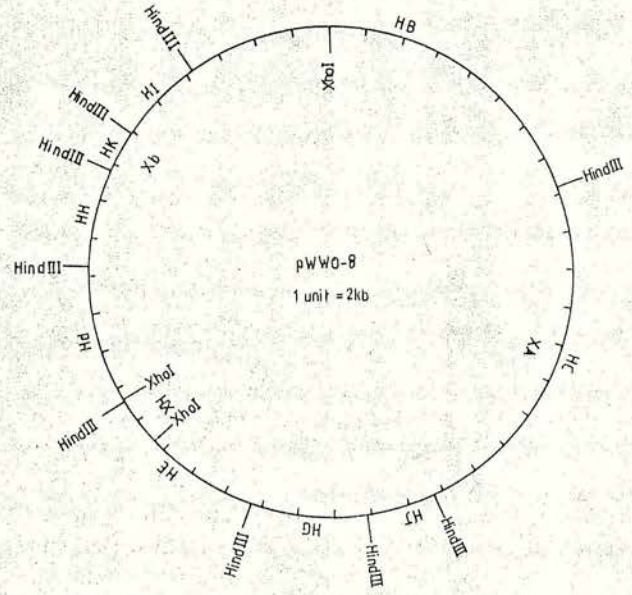

Fig. 2. HindIII and XhoI map of the pWWO-8 plasmid

Table 1. Molecular sizes of restriction fragments

Fragment size (kb)	HindIII	XhoI	HindIII/XhoI
50.6		XA[a]	
26.6		Xb	
22.5	HB		
19.0	HC		HXA
16.2			HXB
8.6	HE		
7.3	Hd		
7.2			HXc
6.4	HG		HXD
6.3			HXE
6.0			HXF
5.2	HH		HXH
4.5	HI		HXK
3.7	HJ		HXM
2.7		XH	
2.6			HXO
2.1	HK		HXR
			HX-

[a] by subtraction

Fragments are lettered in order of size.
Notation used throughout is that of the parental pWWO plasmid. The three fragments unique to pWWO-8 (Hd, Xb and HXc) are given lower case letters.
H = fragment from HindIII digest
X = fragment from XhoI digest
HX = fragment from double digest

The numbers from HindIII and XhoI were both reasonable for map construction. In particular it was striking that pWWO-8 yields only three XhoI fragments; this gave an immediate map. The sizes of all products of single and double digestion with these two enzymes are presented in Table 1. In the subsequent discussion we take the junction of Xb and XA as an arbitrary reference point with XA placed clockwise.

To avoid confusion, the notation used to describe these fragments is based on the number of fragments in the parental pWWO plasmid (the organisation of which is still being studied). Plasmid pWWO-specific fragments HA, HD and HF are absent from the pWWO-8 map and one new fragment (Hd) is present. In the XhoI and double digests, Xb (see above) and HXc are the novel fragments. The notation is described fully in Table 1.

A number of assignments can be made by inspection of fragments sizes and deduction of which ones were contiguous in the intact molecule. Only those assignments that cannot be arrived at unambiguously by such (in principle) simple means will be discussed further.

Plasmid pWWO-8 gives 9 fragments with HindIII and 3 fragments with XhoI. However, in the double digest only 11 fragments can clearly be seen, suggesting that one HX fragment is very small. All three Xho I fragments disappear after digestion by HindIII and therefore each fragment must carry at least one HindIII site. It was found that the three HindIII fragments HB, HE and Hd were absent in the double digest. The novel fragments were HXB, HXc, HXE, HXF and HXO. In addition there is the proposed small fragment (HX-).

Analysis of these data indicate that HE and Hd are adjacent. If the assumption is made that pWWO yielded pWWO-8 by excision of a single segment, we can infer that Hd and Xb (present in the pWWO-8 digests only) include this excision site. Since Hd and

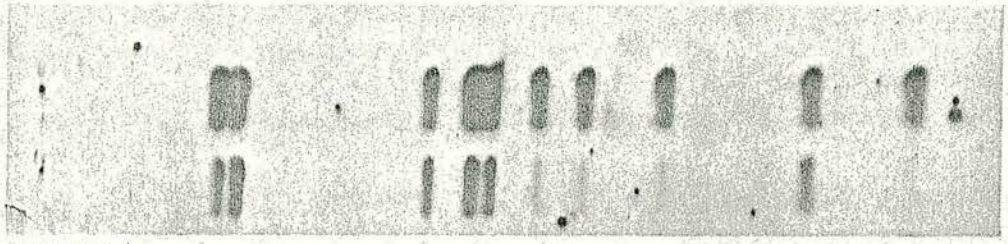

Fig. 3. Autoradiogram showing terminally-labelled fragments from HindIII and XhoI double digests. Lane 1 shows fragments which were labelled after XhoI digestion. In addition to bands HXB, HXc, HXE, HXF and HXO, a number of other faint bands can be seen. These result from incomplete inactivation of the polymerase prior to the second digestion. Lane 2 shows all the double digest products

not HE is similar to Xb in being the unique fragment present in pWWO-8 but not pWWO, Hd must overlap Xb; this establishes the order of Hd and HE as shown in Fig. 1.

Fragment HB might also be located in one of two ways – either the 6.3 kb portion (HXE) or the 16.2 kb portion (HXB) yielded by XhoI digestion could overlap with Xb. This question and others were resolved by measuring the sizes of HindIII partial digestion products. One doublet was HB-HC, showing that these two fragments lie adjacent in the intact plasmid. The existence of a number of others (HJ-HG; HG-HE; HE-Hd; Hd-HH; HH-HK; and HK-HI) gave the order HJ-HG-HE-Hd-HH-HK-HI. Other digestion products were apparently triplets, whose sizes were consistent with this order. On the basis of this proposal the 6.3 kb portion (HXE) of HB overlaps with Xb. The final orders of fragments on pWWO-8 are given in Fig. 2.

As stated earlier, a region present in pWWO but not in pWWO-8 would be bounded on either side by a portion of pWWO-8 HXc. Where the excision event giving rise to pWWO-8 occurred, together with a map of the TOL-specific region of pWWO, are being determined by other means (Villems et al., 1978) and will be reported elsewhere.

We have confirmed the orders of fragments in pWWO-8 given in Fig. 2 by analysis of the patterns obtained from autoradiograms of terminally-labelled single and double digest products. In one experiment the fragments resulting from digestion by XhoI were terminally-labelled prior to digestion by HindIII. As predicted on the basis of our map, only fragments HXB, HXc, HXE, HXF and HXO in the double digest were labelled (but see Fig. 3).

The results of another experiment, in which HindIII fragments were terminally-labelled before digestion by XhoI, were also consistent with the proposed map.

The presence of the smallest fragment (HX-) has not yet been demonstrated directly; however the observation that the 7.3 kb HindIII fragment appears to be labelled at one end only after Xho digestion is strong evidence for its existence.

Terminal-labelling of DNA fragments is commonly carried out using the polynucleotide kinase reaction (Murray, 1973). The techniques described in this paper can be used for a wide range of endonuclease generated fragments which have 'cohesive ends'.

Although not applicable in the present study, cohesive ends generated by a particular endonuclease can be distinguished in a mixture of restriction fragments provided they differ in composition by at least one deoxyribonucleotide residue. Thus for example, EcoRI-generated ends (-TTAA) can be distinguished from those of HindIII (-TCGA) during a labelling experiment if the labelled deoxyribonucleotide triphosphate is dGTP or dCTP.

Acknowledgements. R.V. was a visiting British Council Scholar from Tartu University, Estonia, USSR.

References

Bayley, S.A., Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G., Broda, P.: Two modes of loss of the Tol function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154**, 203–204 (1977)

Duggleby, C.J., Bayley, S.A., Worsey, M.J., Williams, P.A., Broda, P.: Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. **130**, 1274–1280 (1977)

Hansen, J.B., Olsen, R.H.: Isolation of large bacterial plasmids and characterisation of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**, 227–238 (1978)

Murray, K.: Nucleotide sequence analysis with polynucleotide kinase and nucleotide 'mapping' methods. Biochem. J. **131**, 569–583 (1973)

Villems, R., Duggleby, C.J., Broda, P.: Restriction endonuclease mapping of DNA using *in situ* digestion in two-dimensional gels. FEBS Lett. **89**, 267–270 (1978)

Communicated by E. Bautz

Received September 26, 1978

Molec. gen. Genet. 177, 189–191 (1979)

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Short Communication

A Cleavage Map of the TOL Plasmid of *Pseudomonas putida* mt-2

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Summary. A cleavage map of the TOL plasmid pWWO has been determined for the restriction endonucleases *Hin*dIII and *Xho*I. A number of techniques were employed including (i) digestion of purified cleavage products with a second enzyme; (ii) hybridisation of purified *Xho*I fragments to Southern blots of *Hin*dIII digest products and (iii) analysis of a number of deletion mutants.

The TOL plasmid pWWO of *P. putida* mt-2 is a transmissible plasmid 117 kilobases in length which carries genes specifying the degradation of the aromatic hydrocarbons toluene, *m*-xylene and *p*-xylene. The loss of this degradative function for example in pWWO-8, has been correlated with the loss of a specific segment of approximately 40 kb (Bayley et al., 1977). Recently a number of RP4-TOL recombinants have been isolated (Chakrabarty et al., 1978; Jacoby et al., 1978; Nakazawa et al., 1978); in suitable hosts these express both the antibiotic resistance and the degradative functions. It has been estimated that in each case about 57 kb of pWWO DNA has been acquired by the RP4 molecule. A physical map of the pWWO plasmid is necessary in order to relate these two observations.

Because of the number of fragments and the wide range in their sizes (Table 1) a combination of techniques proved necessary, firstly to visualise the fragments and secondly to obtain an order. Plasmid DNA was routinely labelled with ^{32}P dGTP before use either by nick-translation or by terminal labelling (Downing et al., 1979). This latter technique was particularly useful since all fragments could then be visualised equally well. Information on the order of fragments was obtained using the following techniques:

(1) digestion by *Hin*dIII of purified *Xho*I fragments recovered from gels,

(2) hybridisation of purified fragments recovered from gels to Southern transfers of restriction digests (Chilton et al., 1978),

(3) analysis of a number of deletion mutants generated by Tn401 (Benson and Shapiro, 1978).

Where it was not immediately apparent, the internal order of, for example, several *Xho*I fragments within a single *Hin*dIII fragment was deduced from analysis of partial digest products. In a number of cases the derived order of fragments has been confirmed by analysis of clones of *Hin*dIII fragments in the *E. coli* vector pBR322 (unpublished).

The methods used are described in Fig. 1 or in a previous publication (Downing et al., 1979), which also describes the derivation of the cleavage map for pWWO-8.

The first step in constructing a map was to determine which two fragments form the ends of the 40 kb segment (the fragments concerned are shown in Table 2). In the deleted plasmid pWWO-8, there is one novel fragment (Hd); this is composed of DNA left over from these two fragments after excision has occurred. To identify these fragments ^{32}P dGTP-labelled Hd was recovered from a gel (see Fig. 1 for details) and used as a probe against Southern transfers of pWWO digested with *Hin*dIII. This established that the ends of the 40 kb segment were formed by HD and HF.

The orientation of the segment with respect to pWWO-8 was not apparent. We therefore first considered the internal order of the 9 *Hin*dIII fragments. Three of these (HA, HD and HF) are missing in the double digest, i.e. they carry sites for *Xho*I. Re-

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Table 1. Molecular sizes of restriction fragments from pWWO

Fragment size (kilobases)	*Hin*dIII	*Xho*I	*Hin*dIII/*Xho*I
51		XA[a]	
25		Xb	
25	*HA*		
23		*XB*	
22	HB		
19	HC		HXA
16		XC	
16			HXB
11.8			*HXC*
10.0	*HD*		
8.4	HE		
7.3	Hd		
7.2	*HF*		
7.2			HXc
6.5		*XD*	
6.2			HXD
6.1	HG		HXE
5.8			HXF
5.3		*XE*	*HXG*
5.0	HH		HXH
4.7		*XF*	*HXI*
4.7			*HXJ*
4.4	HI		HXK
4.2		*XG*	*HXL*
3.6	HJ		HXM
3.6			*HXN*
2.7		XH	
2.6			HXO
2.30			*HXP*
2.25		*XI*	*HXQ*
2.10	HK		HXR
1.56		*XJ*	*HXS*
1.23	*HL*		*HXT*
1.03	*HM*		*HXU*
0.79			*HXV*
0.60	*HN*		*HXW*
0.55	*HO*		*HXX*
0.50	*HP*		*HXY*
0.50	*HQ*		*HXZ*
0.28	HR		HXA1
0.20	HS		HXB1
0.13			HXC1

Fragments are lettered in order of size
The three fragments unique to pWWO-8 are lettered in lower case
Fragments italicized are unique to the parental plasmid
H = fragment from *Hin*dIII digest
X = fragment from *Xho*I digest
HX = fragment from double digest

[a] By subtraction

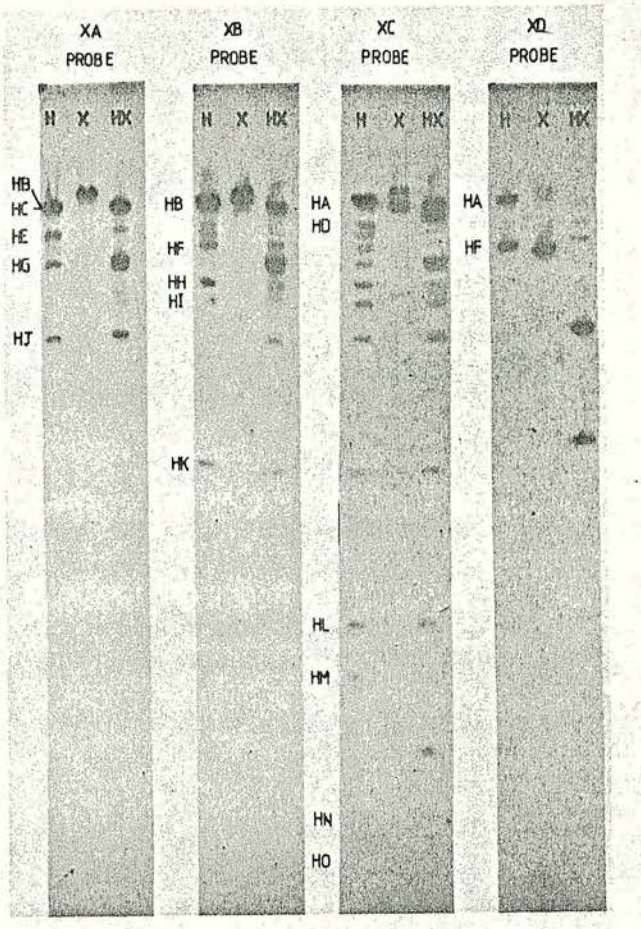

Fig. 1. Autoradiograms of purified DNA of the four largest X fragments (used as probes) hybridised against Southern transfers of *Hin*dIII, *Xho*I and double-digest (HX) fragments. The probes were prepared from ^{32}P dGTP-labelled *Xho*I fragments of pWWO cut from a 0.5% agarose gel and recovered by electroelution in 5 mM Tris-acetate buffer pH 6.0. The DNA was denatured for 5′ at 100° and hybridisation against Southern transfers of *Hin*dIII and *Xho*I digests of pWWO carried out according to the method of Dawid (1977). The fragments on the filter that are within or overlap the probe fragments are lettered. Hybridisation with other fragments, especially where XB was the probe, was due to imperfect resolution of the probe DNA in the original purifications

gardless of the number of such sites on any one *Hin*dIII fragment there will be only *2* novel fragments for each of the 3 *Hin*dIII fragments in the double digest, i.e. a total of 6. The approach we used to obtain a map was to determine which of the six novel fragments were contained within each of the three *Hin*dIII fragments. In some cases this was apparent from the hybridisation data (Fig. 1); in others it was obvious from a consideration of the molecular weight data, e.g. HXC (11.8 kb) could only be contained by HA (23 kb).

Having established which fragments formed the ends of HA, HD and HF and of XB, XC and XD it was then possible to establish the overlap between *Xho*I fragments and *Hin*dIII fragments. Thus HXC is contained by HA and by XC, i.e. HA overlaps XC by the length of HXC. This approach enabled

Table 2. Restriction fragments found in digests of pWWO but not pWWO-8

Fragment size (kilobases)	*Hin*dIII	*Xho*I	*Hin*dIII/*Xho*I
25	(HA)		
23		(XB)	
16		(XC)	
11.8			HXC*
10.0	(HD)		
7.2	(HF)		
6.5		(XD)	
5.3		XE	HXG
4.7		XF	HXI
4.7			HXJ*
4.2		XG	HXL
3.6			HXN*
2.30			HXF*
2.25		XI	HXQ
1.56		XJ	HXS
1.23	HL		HXT
1.03	HM		HXU
0.79			HXV*
0.60	HN		HXW
0.55	HO		HXX
0.50	HP		HXY
0.50	HQ		HXZ
0.13			HXC1 [a]*

[a] HXC1 (which is also present in pWWO-8) is included here since it is part of HD or HF

The fragments in parentheses carry sites for the other enzyme since they are not present in the double digest

There are 6 fragments in the double digest (*) which are not found in either single digest

us to place HA, HD and HF in order. Mapping of the remaining fragments was achieved by a consideration of their molecular sizes in conjunction with other information on partial digest products and deletion mutants (Benson and Shapiro, 1978). Finally because fragment HXC1 (which has already been mapped in pWWO-8) is part of HD and not HF, the orientation of the 40 kb segment with respect to the remainder of pWWO-8 is as shown in Fig. 2.

It can be seen from Table 1 that the distribution of cleavage sites for *Hin*dIII is markedly non-random. It is of interest that many of these cleavage sites are clustered together (Fig. 2).

Acknowledgements. We thank Clive Duggleby, Richard Villems and Saveria Campo for discussions and the Science Research Council for support.

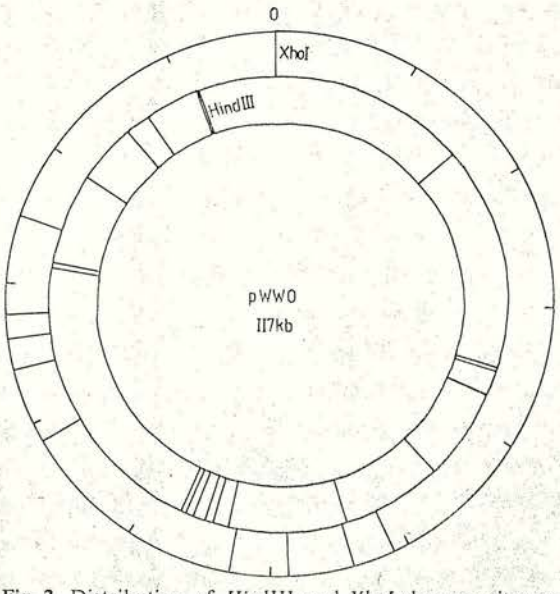

Fig. 2. Distribution of *Hin*dIII and *Xho*I cleavage sites on TOL. The site between fragments XA and XB is used as a reference point. The region absent in pWWO-8 runs from about 55 kb to 95 kb, reading clockwise. The precise order of several small *Hin*dIII fragments has not been determined except that on the basis of hybridisation data they lie in two distinct groups

References

Bayley, S.A., Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G., Broda, P.: Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. **154**, 203–204 (1977)

Benson, S., Shapiro, J.: TOL is a broad-host-range plasmid. J. Bacteriol. **135**, 278–280 (1978)

Chakrabarty, A.M., Friello, D.A., Bopp, L.H.: Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. Proc. Natl. Acad. Sci. U.S.A. **75**, 3109–3112 (1978)

Chilton, M.-D., Montoya, A.L., Merlo, D.J., Drummond, M.H., Nutter, R., Gordon, M.P., Nester, E.W.: Restriction endonuclease mapping of a plasmid that confers oncogenicity upon *Agrobacterium tumefaciens* strain B6-806. Plasmid **1**, 254–269 (1978)

Dawid, I.B.: DNA-DNA hybridisation on membrane filters: a convenient method using formamide. Biochim. Biophys. Acta **477**, 191–194 (1977)

Downing, R.G., Duggleby, C.J., Villems, R., Broda, P.: An endonuclease cleavage map of the plasmid pWWO-8, a derivative of the TOL plasmid of *Pseudomonas putida* mt-2. Mol. Gen. Genet. **168**, 97–99 (1979)

Jacoby, G.A., Rogers, J.E., Jacob, A.E., Hedges, R.W.: Transposition of *Pseudomonas* toluene-degrading genes and expression in *Escherichia coli*. Nature **274**, 179–180 (1978)

Nakazawa, T., Hayashi, E., Yokota, T., Ebina, Y., Nakazawa, A.: Isolation of TOL and RP4 recombinants by integrative suppression. J. Bacteriol. **134**, 270–277 (1978)

Communicated by E. Bautz

Received September 19, 1979

JOURNAL OF BACTERIOLOGY, Dec. 1982, p. 1280–1283
0021-9193/82/121280-04$02.00/0

Vol. 152, No. 3

Physical Mapping of TOL Plasmids pWWO and pND2 and Various R Plasmid-TOL Derivatives from *Pseudomonas* spp.

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Received 4 January 1982/ Accepted 6 July 1982

Analysis of several independently isolated R plasmid-TOL hybrids revealed a wide variation in the amount of TOL DNA they contain. If the formation of the various R plasmid-TOL hybrids involves transposition (which has yet to be rigorously assessed), such transposition does not involve a unique segment of TOL DNA.

The TOL plasmid of *Pseudomonas putida* mt-2 confers on its host the ability to utilize toluene and several toluene derivatives (11, 16, 17). A physical map of the archetypal TOL plasmid, pWWO, has been published (3), and several functions have been mapped on the restriction enzyme map of pWWO (6) and of an RP4-TOL derivative (13). Early in the analysis of the TOL (pWWO) plasmid, it was shown that recombinants between R plasmids and pWWO could be isolated (2, 14, 15). This has been explained as "transposition" of segments of the pWWO plasmid into the R plasmids studied (2, 10). However, it has not been rigorously demonstrated whether the recombinant formation fulfills the criteria of true transposition.

This paper describes the isolation of a new R plasmid-TOL recombinant and the physical analysis of this and several other R plasmid-TOL recombinants. The new R plasmid-TOL hybrid is a recombinant between the Inc-W group plasmid Sa (9, 14a) and the Tol plasmid pND2 (15). The plasmid pND2 was isolated by White and Dunn (15) after a plate mating between *P. putida* PAri-6 (TOL) and *Pseudomonas aeruginosa* PAO3 (*trp*$^-$). The ability of PAO3 exconjugants to grow on *m*-toluate was rapidly lost under nonselective conditions (growth in Luria broth); however, a stable TOL$^+$ variant, strain PAO3 (pND2), was isolated (15). We have compared pND2 with pWWO by using the enzymes *Eco*RI, *Hin*dIII, and *Xho*I and have seen no differences between them. No gross structural changes, therefore, are associated with the stability of the TOL plasmid pND2 in *P. aeruginosa* PAO. The ability to degrade *m*-toluate was readily transferred from *P. aeruginosa* PAO3 pND2 to *P. putida* AC34 (2×10^{-2} transconjugants per donor cell), whereas transfer to other strains of *P. aeruginosa* PAO was not observed ($<10^{-6}$ transconjugants per donor cell). This suggests that the stability of pND2 in *P. aeruginosa* PAO is due to a host mutation(s). Curing and reinfection experiments would be necessary to test this hypothesis.

An earlier report (2) also describes the isolation of PAO (Tol$^+$) exconjugants. In this case the strains were unable to transfer the TOL plasmid (termed TOL*) to either *P. aeruginosa* or *P. putida* recipients. TOL* is also considerably smaller (28 megadaltons) than the original TOL plasmid (2). These properties thus differ from those of pND2; TOL* and pND2 therefore may reflect different mechanisms by which TOL is established in *P. aeruginosa* PAO.

To allow more detailed analyses of pWWO, pND2, and the various R plasmid-TOL derivatives, we have constructed an *Eco*RI cleavage map of pWWO. *Eco*RI digests of pWWO DNA give 29 fragments (5), compared with the *Hin*dIII (19 fragments) and *Xho*I (10 fragments) digests (3). The *Eco*RI map was deduced from a series of experiments with double (*Hin*dIII, *Eco*RI and *Xho*I, *Eco*RI) digests of individually cloned *Hin*dIII fragments of pWWO (unpublished data). Bacteriophage lambda DNA digested with *Hin*dIII and pWWO DNA digested with *Hin*dIII and *Xho*I (3) were used as DNA size markers.

The new R plasmid-TOL recombinant was isolated as detailed below. A loopful of PAO3 (pND2, Sa) cells that had been grown overnight in nutrient broth at 42°C was used in a plate mating with *P. aeruginosa* PAO2 (*ser*$^-$). Co-transfer of a drug resistance marker (gentamicin

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resistance) and the Tol$^+$ phenotype were selected for by plating on minimal medium supplemented with serine (20 μg ml^{-1}), *m*-toluate (10 mM), and gentamicin (20 μg ml^{-1}). From the colonies that appeared, a clone containing an Sa-TOL hybrid plasmid (pMT101) was obtained.

The R plasmid-TOL recombinants studied are shown in Fig. 1. Purified plasmid DNA preparations from *P. putida* PU21 (pED3304), an RP4-TOL recombinant (10), *P. aeruginosa* PAO3 (pND3), an R91-TOL recombinant (15), and *P. aeruginosa* PAO2 (pMT101), an Sa-TOL recombinant, were restricted with *Eco*RI, *Hin*dIII, and *Xho*I and compared with similar digests of pWWO or pND2. The plasmid pED3304 has a continuous 69-kilobase region of TOL inserted into a single site in RP4 within the tetracycline resistance gene. The *Hin*dIII digests show that fragments HB, HC, HS, HR, HJ, and HG are missing or incomplete. The endpoints of TOL DNA could now be more accurately mapped with reference to the *Eco*RI digests; one endpoint within HG occurs within the 1.8-kilobase region between the end of the *Eco*RI EW fragment (intact in pED3304) and the end of HG. The other endpoint is within the small *Hin*dIII fragment, HS.

The R91-TOL hybrid, pND3, contains the largest continuous segment of TOL DNA (approximately 104 kilobases); all the TOL *Hin*dIII fragments except HC, HG, HJ, and HR are present. The endpoint within HG appears to be similar to that of pED3304; the other endpoint, within HC, is in *Eco*RI fragment ER or EJ.

Digests with *Hin*dIII show that the Sa-TOL hybrid, pMT101, and the RP4-TOL hybrid, pTN2, contain the smallest segment of TOL DNA among the plasmids studied (approximate-

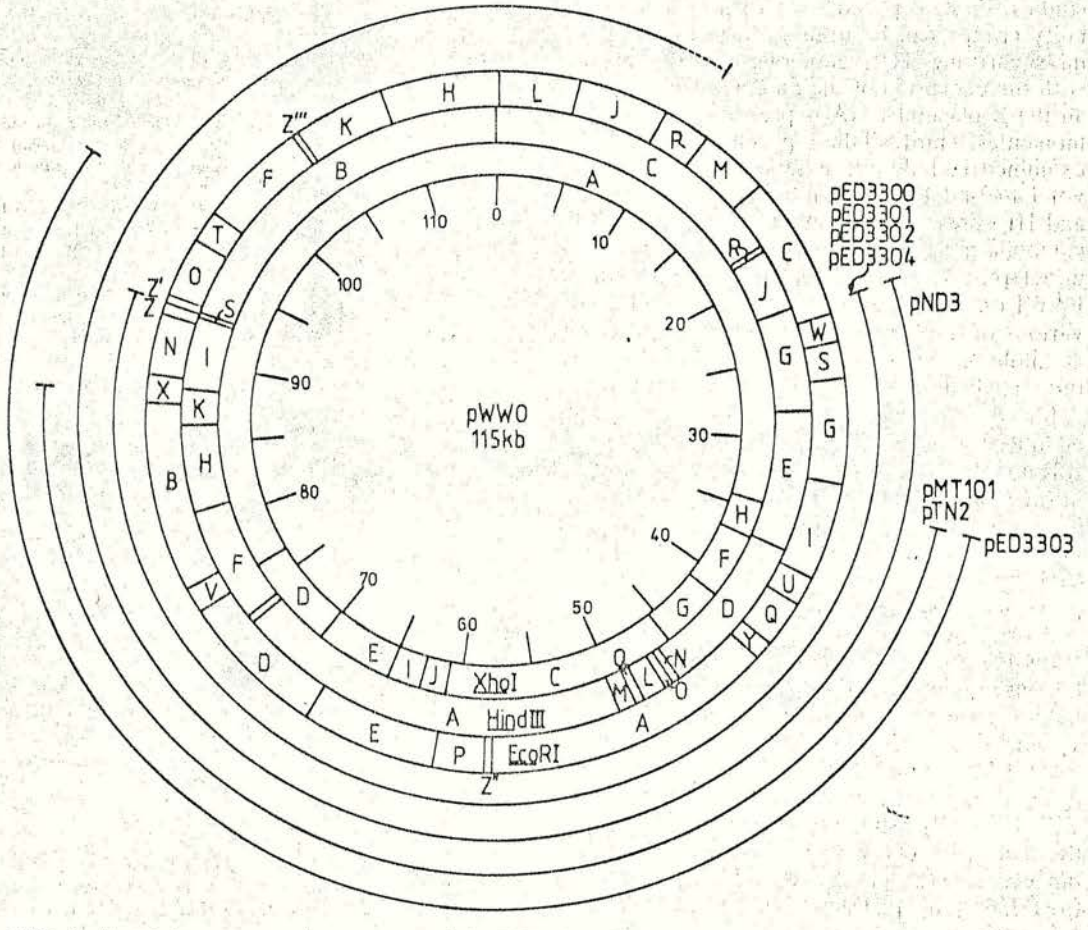

FIG. 1. Restriction enzyme cleavage map of the TOL plasmid pWWO (and pND2). Fragments produced by each enzyme (*Eco*RI, *Hin*dIII, and *Xho*I) are labeled alphabetically in order of decreasing size. The extent of TOL DNA present in various R plasmid-TOL derivatives is indicated by solid continuous lines. One end of pND3 is dotted to indicate that the endpoint of TOL DNA is in *Eco*RI fragment EJ or ER. The numbers on the inner ring represent kilobases (kb).

ly 56 kilobases). The *Hin*dIII fragments HB, HC, HR, HJ, HG, HE, HS, HI, and HK are missing or incomplete. One endpoint is within the TOL *Eco*RI EI fragment (and HE), and the other endpoint is within HK (and *Eco*RI EX). Interestingly, pMT101 lacks 5.4 kilobases of Sa and consequently chloramphenicol resistance. However, this type of deletion event has been reported before for Sa (8) and thus may not be related to the formation of the Sa-TOL hybrid.

Data from several other independently isolated RP4-TOL plasmids have recently been summarized (12). Further analysis of *Eco*RI digests of these hybrids (pED3300, pED3301, pED3302, and pED3303) allowed accurate determination of the amounts of TOL DNA that they carried. Also included in this examination was the previously characterized RP4-TOL plasmid pTN2 (13). The results presented in Fig. 1 allow a comparison of all the plasmids analyzed.

Several conclusions can be drawn from these studies. First, R plasmids of various incompatibility groups can be used to form hybrid plasmids carrying TOL catabolic genes. Second, with the exception of the Sa derivative studied, all the R plasmid DNA is present in the hybrid molecules. Third, all the R plasmid-TOL hybrids examined include and extend at both ends beyond a 40-kilobase region (between *Hin*dIII HD and HF) previously known to contain the TOL catabolic genes (1). This region is bounded by direct repeats (12) and is lost after growth of PAW1 on benzoate (1, 4). Similarly, pND2 and various of the R plasmid-TOL hybrids lose this 40-kilobase region when grown on benzoate (unpublished data).

Finally, the amount of TOL DNA incorporated in R plasmids varied from 56 to 104 kilobases. It is not clear at this stage whether this variation in the amount of TOL DNA reflects differences in the method of selection and isolation of R plasmid-TOL hybrids. In the case of pMT101 (this study) and pTN2 (13, 14), integrative suppression by selecting for the Tol$^+$ phenotype and drug resistance at 42°C in *P. aeruginosa* PAO led to the integration of the same 56-kilobase region of TOL into Sa and RP4, respectively. However, a similar selection for the stabilization of TOL at 37°C in *P. aeruginosa* PAO R91-containing cells produced a hybrid plasmid (pND3) containing 104 kilobases of TOL DNA. By using the method of cotransfer of the Tol$^+$ phenotype and particular drug resistances, several of the RP4-TOL derivatives (pED3304 [10], pED3300 [7], and pED3301 [P. Williams, personal communication]) were independently isolated. In all these cases, a 69-kilobase region of TOL has become incorporated into the RP4 plasmid. Two groups (2, 10) have suggested that transposition of the Tol$^+$ phenotype may be responsible for the formation of RP4-TOL hybrids; however, it is clear from our data that such transposition could not involve a unique segment of TOL DNA. We are currently investigating the junction restriction fragments of the R plasmid-TOLs DNA in order to understand the formation of these hybrids.

This work was supported by grants from the Medical Research Council and the Science and Engineering Research Council to P.B. P.M. was a Medical Research Council scholar.

We are grateful to I. McGregor for technical assistance.

LITERATURE CITED

1. **Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda.** 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. 154:203–204.
2. **Chakrabarty, A. M., D. A. Friello, and L. H. Bopp.** 1978. Transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms. Proc. Natl. Acad. Sci. U.S.A. 75:3109–3112.
3. **Downing, R. G., and P. Broda.** 1979. A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. Mol. Gen. Genet. 177:189–191.
4. **Downing, R. G., C. J. Duggleby, R. Villems, and P. Broda.** 1979. An endonuclease cleavage map of the plasmid pWWO-8, a derivative of the TOL plasmid of *Pseudomonas putida* mt-2. Mol. Gen. Genet. 168:97–99.
5. **Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda.** 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. 130:1274–1280.
6. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta* cleavage pathway. Proc. Natl. Acad. Sci. U.S.A. 78:7458–7462.
7. **Franklin, F. C. H., and P. A. Williams.** 1980. Construction of a partial diploid for the degradative pathway encoded by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2: evidence for the positive nature of the regulation by the *xylR* gene. Mol. Gen. Genet. 177:321–328.
8. **Goral, A. P., F. Heffron, S. Falkow, R. W. Hedges, and N. Datta.** 1979. Electron microscope heteroduplex studies of sequence relationships among plasmids of the N incompatibility group. Plasmid 2:485–492.
9. **Hedges, R. W., and N. Datta.** 1971. Fi$^-$ R factors giving chloramphenicol resistance. Nature (London) 234:220–221.
10. **Jacoby, G. A., J. E. Rogers, A. E. Jacob, and R. W. Hedges.** 1978. Transposition of *Pseudomonas* toluene-degrading genes and expression in *Escherichia coli*. Nature (London) 274:179–180.
11. **Kunz, D. A., and P. J. Chapman.** 1981. Catabolism of pseudocumene and 3 ethyltoluene by *Pseudomonas putida* (*arvilla*) mt-2: evidence for new functions of the TOL plasmid. J. Bacteriol. 146:179–191.
12. **Meulien, R., R. G. Downing, and P. Broda.** 1981. Excision of the 40kb segment of the TOL plasmid from *P. putida* mt-2 involves direct repeats. Mol. Gen. Genet. 184:97–101.
13. **Nakazawa, R., S. Inouye, and A. Nakazawa.** 1980. Physical and functional mapping of RP4-TOL plasmid recombinants: analysis of insertion and deletion mutants. J. Bacteriol. 144:222–231.
14. **Nakazawa, T., E. Hayashi, T. Yokota, Y. Ebina, and A.**

Nakazawa. 1978. Isolation of TOL and RP4 recombinants by integrative suppression. J. Bacteriol. 134:270–277.

14a. Ward, J. M., and J. Grinsted. 1982. Physical and genetic analysis of the IncW group plasmids R338, Sa and R7K. Plasmid 7:239–250.

15. White, G. P., and N. W. Dunn. 1977. Apparent fusion of the TOL plasmid with the R91 drug resistance plasmid in *Pseudomonas aeruginosa*. Aust. J. Biol. Sci. 30:345–355.

16. Williams, P. A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. 120:416–423.

17. Wong, C. L., and N. W. Dunn. 1974. Transmissible plasmid coding for the degradation of benzoate and *m*-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. 23:227–232.

JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 911–914
0021-9193/82/110911-04$02.00/0

Vol. 152, No. 2

Identification of Chromosomally Integrated TOL DNA in Cured Derivatives of *Pseudomonas putida* PAW1

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Received 21 May 1982/Accepted 20 July 1982

Some plasmid-free Tol⁻ strains derived from *Pseudomonas putida* PAW1 (which carries the TOL plasmid pWW0) have a segment of TOL DNA located chromosomally. Of three independently isolated strains, PAW86 had an integrated TOL segment of 16 kilobases and PAW85 had two copies of this segment in different chromosomal locations, whereas the chromosomal DNA of PAW82 showed no homology with the TOL plasmid. In cultures of the parental strain, it appears that a 56-kilobase TOL DNA segment is located chromosomally in some cells.

TOL plasmid pWW0 from *Pseudomonas putida* mt-2 (strain PAW1) (Table 1) encodes the enzymes necessary for the degradation of toluene and the *m*- and *p*-xylenes via the *meta* cleavage pathway (12, 13). Strains lacking this degradative function can be selected after growth on benzoate, an intermediate in both the plasmid-encoded *meta* pathway and the chromosome-encoded *ortho* pathway (13). Such benzoate curing can occur either by loss of the plasmid from the cell or by specific excision of a 40-kilobase (kb) segment of the plasmid which results in the formation of the Tol⁻ plasmid pWW0-8 (1). We have previously shown that the latter event occurs due to reciprocal recombination between a pair of directly repeated sequences present in the *Hin*dIII fragments HD and HF at the ends of the 40-kb segment. A new *Hin*dIII fragment, Hd, is thus formed, it being a hybrid of fragments HD and HF (8).

After the chance observation that DNA from a strain (PAW86) that had lost pWW0 after benzoate selection showed homology with the TOL plasmid in DNA-DNA hybridization studies (D. Morris and P. Broda, unpublished data), we analyzed the chromosomal DNA of strain PAW86 and two other independently isolated plasmid-free derivatives of strain PAW1 (strains PAW82 and PAW85) (Table 1) to assess the amount of pWW0 DNA present chromosomally in each.

DNA from these strains was isolated by the method of Dhaese et al (4), digested with endonuclease *Xho*I (Bethesda Research Laboratories), run out on 0.7% agarose gels, transferred to nitrocellulose, and hybridized to pWW0 DNA that had been ^{32}P labeled by nick translation (10). DNA from the parent strain PAW1 was used as the positive control, and that from the plasmid-free strain AC34 was used as the negative control. Some homology with TOL was observed with the DNA from strains PAW85 and PAW86 but not with the DNA from strain PAW82 (Fig. 1). We next tried to establish to which segments of the TOL plasmid these regions corresponded.

Inspection of the data presented in Fig. 1 allows comparison of the homology to pWW0 observed with the DNA from strains PAW85 and PAW86 with that of the pWW0-containing strain PAW1. This shows that pWW0 segments bounded by most of the *Xho*I target sites could not be present in the DNA from the two derivative strains in the form in which they exist in pWW0 itself. We can exclude the presence of the segment that includes fragments XF, XG, XC, XJ, XI, XE, and XD. (The presence of *Hin*dIII fragment Hd in the chromosomal DNA of these strains excludes the possibility of *Xho*I fragment XC being present.) This continuous region includes the *Hin*dIII fragment HA and approximates the 40-kb segment excised by benzoate selection (Fig. 2). The presence or absence of the remainder of the pWW0 genome was tested in hybridization experiments with isolated *Hin*dIII-generated fragments as specific probes.

DNA samples from strains PAW82, PAW85, and PAW86 were digested with *Hin*dIII, run out on agarose gels, transferred to nitrocellulose filters, and hybridized to specific *Hin*dIII fragments of TOL. These fragments, with the exceptions of HB and HH, had been obtained as clones in the vector plasmid pBR322 (2) with

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TABLE 1. Strains used

Strain	Plasmid	Plasmid size (kb)	Cloned *Hin*dIII fragment[a]	Reference
P. putida				
PAW1	pWW0	115		12
PAW8	pWW0-8	75		1
PAW82, PAW85, and PAW86				1
AC34				6
Escherichia coli				
ED3306	pED3306	14.4	HD	8
ED3307	pED3307	11.6	HF	8
ED3309	pED3309	23.4	HC	This study
ED3310	pED3310	12.8	HE	This study
ED3311	pED3311	10.1	HG	This study
ED3312	pED3312	8.8	HI	This study
ED3313	pED3313	8.0	HJ	This study
ED3314	pED3314	6.5	HK	This study

[a] All *Hin*dIII fragments were cloned into pBR322, which is 4.3 kb (2).

*Hin*dIII-restricted pWW0 DNA (Table 1; R. Downing and P. Broda, unpublished data). The various probes were made by digesting 2 μg of each recombinant plasmid with *Hin*dIII, separating the fragments from the vector DNA on agarose gels, cutting out the gel slice containing the relevant fragment, and extracting the DNA according to the method of Thuring et al. (11). This DNA was then ^{32}P labeled by nick translation. In the cases of fragments HB and HH, the fragments were obtained after *Hin*dIII digestion of pWW0 DNA and subsequent separation on agarose gels.

On hybridization, DNA from strain PAW86 showed homology with probes from five *Hin*dIII fragments, HD (10.0 kb), HE (8.0 kb), HF (7.2 kb), HH (5.0 kb), and HK (2.7 kb). The sizes of the respective hybridizing fragments from strain PAW86 were 7.2, 6.3, 7.2, 5.0, and 1.5 kb (Fig. 3 shows the results for fragments HE and HK). No hybridization was observed with *Hin*dIII fragments HB, HC, HJ, HG, and HI (data not shown). The 5.0- and 7.2-kb chromosomal fragments are likely to be of purely pWW0 origin (fragments HH and Hd, respectively), but the 6.3- and 1.5-kb fragments must be composite, as would be expected if a segment of pWW0, the ends of which are located in the *Hin*dIII fragments HE and HK, is integrated in the chromosome of strain PAW86.

When the same experiments were performed on DNA from strain PAW85, *Hin*dIII fragments HD, HF, HH, HE, and HK all gave positive results again, and negative results were obtained as with strain PAW86. The interesting observation, however, was that fragments HK and HE each showed strong homology with two *Hin*dIII fragments. In each case, one of the two fragments was identical in size to that hybridizing in strain PAW86 with the appropriate probe (Fig. 3). The same interpretation of the results obtained with strain PAW86 DNA is valid here except that two copies of the integrated segment appear to be present at different chromosomal

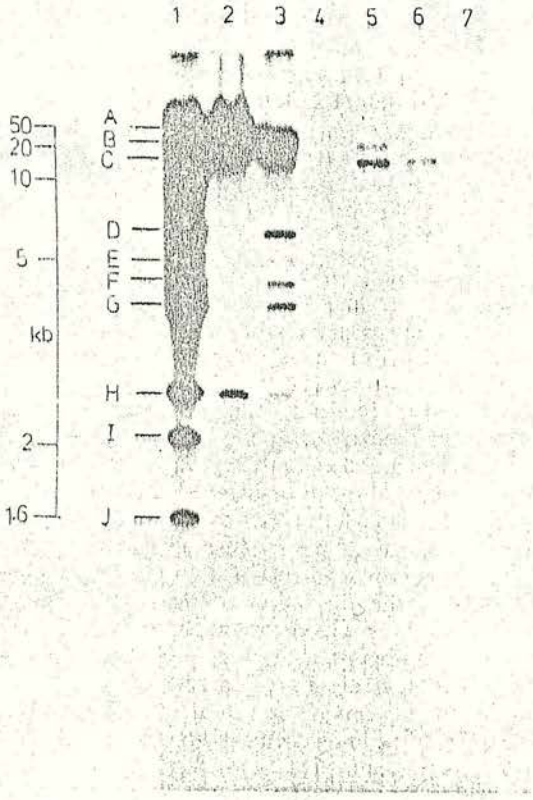

FIG. 1. Southern blots of *Xho*I-restricted chromosomal DNA with pWW0 as a labeled probe. Lanes 1 and 2 contain purified pWW0 and pWW0-8 DNA, respectively. Lanes 3 through 7 contain total cellular DNA from strains PAW1 (grown on *m*-toluate) (lane 3), PAW82 (lane 4), PAW85 (lane 5), PAW86 (lane 6), and AC34 (lane 7). In lane 6, *Xho*I fragment H of pWW0 was visible on the original autoradiogram. The different amounts of hybridization observed for certain fragments in lanes 5 and 6 reflect small differences in the quantities of DNA samples loaded on the gel. Based on the data presented in Fig. 3, two novel fragments in lane 6 should have hybridized to the pWW0 probe. The fact that only one is seen here suggests that the second may be very small, therefore running off the gel, or so large that its transfer onto the filter would be very inefficient. In lane 3, bands XG and XD are more intense due to the repeated DNA sequence present within *Hin*dIII fragments HD and HF.

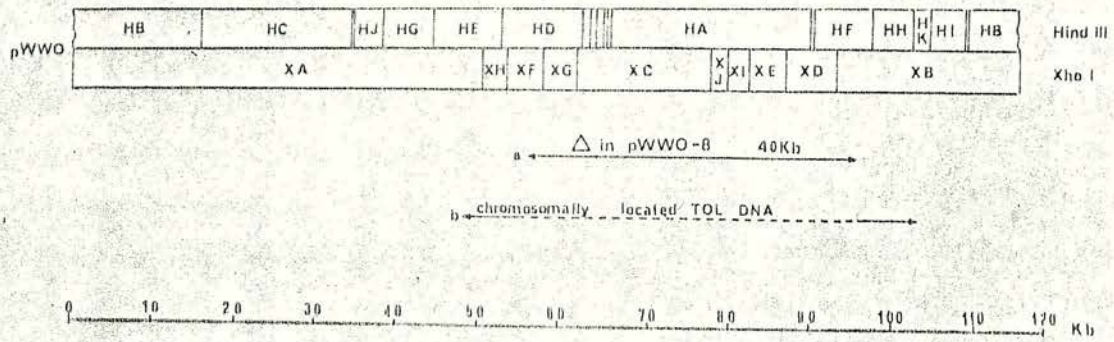

FIG. 2. *Hin*dIII and *Xho*I cleavage maps of pWW0 showing the specific deletion occurring in pWW0-8 formation (a) and the endpoints of the integrated segment in strains PAW85 and PAW86 (b). The *Xho*I cleavage map differs from that previously published (5) in that the positions of fragments XE and XI are reversed. The dotted portion of line (b) indicates the DNA which is absent in the cured derivative strains PAW85 and PAW86. This corresponds in size and location to the 40-kb excised segment represented by line (a). The solid portion of line (b) shows the DNA which is present in the cured derivatives.

locations; one would be at the same site as in strain PAW86.

As stated above, *Hin*dIII digests of total cellular DNA from strain PAW1 were routinely included as positive controls in the different hybridizations with the specific *Hin*dIII fragments as probes. In general, the expected results were obtained; namely, homology was observed only with the single fragment used as the probe. However, in the cases of fragments HE and HK, homology to additional fragments (6.3 and 1.6 kb, respectively) was observed. These fragments are not carried by the pWW0 plasmid itself, and we conclude that in a significant proportion of cells of our culture of the parental strain, PAW1, there is also pWW0 DNA at a particular chromosomal location.

This segment could be a continuous region of the pWW0 genome containing all of the DNA sequences between *Hin*dIII fragments HK and HE (56 kb). However, it was not possible to demonstrate this by the experiments described here. Such a model in turn suggests that the integrated segment in strains PAW85 and PAW86 is the 16 kb of DNA that remains after the excision of the internal 40-kb portion of the 56-kb segment. The results obtained with strain PAW85 suggest that a second copy of this region can be maintained at a second location. The lack of any pWW0 DNA in the chromosome of strain PAW82 could be explained by the straightforward loss of the entire plasmid in this particular curing event.

A similar segment of 56 kb of pWW0 DNA has

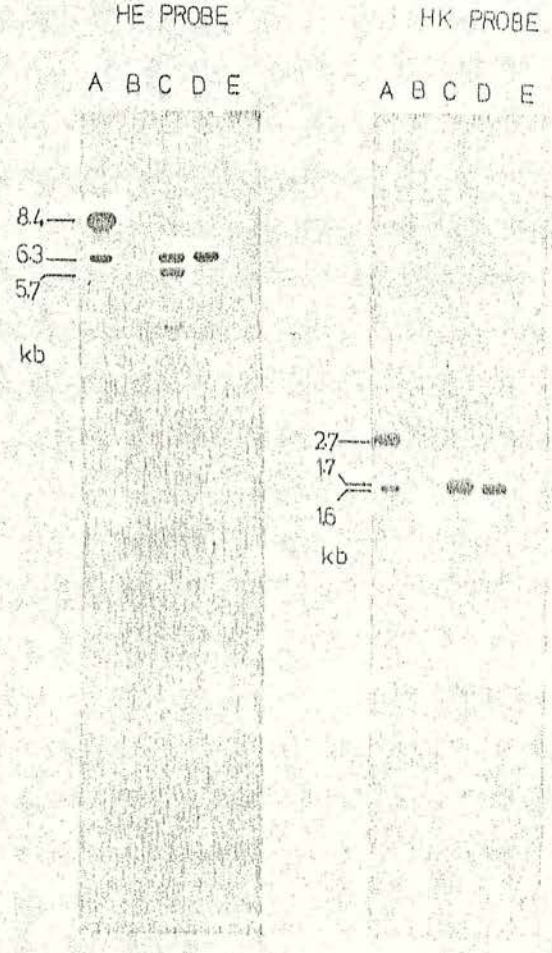

FIG. 3. Southern blots of *Hin*dIII-restricted chromosomal DNA with fragments HE and HK as probes. Lanes: A, strain PAW1; B, strain PAW82; C, strain PAW85; D, strain PAW86; E, strain AC34. The strongly hybridizing band in lane C of the HK probe is a doublet. The faint bands visible in lanes C and E of the HE probe and lanes C and D of the HK probe may reflect some homology between the probes and more distantly related chromosomal sequences; however, we cannot discount the possibility that this observation is due to nonspecific hybridization.

been implicated in recombination events which superficially resemble transposition involving TOL and plasmids RP4 (9), Sa (P. R. Lehrbach, J. M. Ward, P. Meulien, and P. Broda, J. Bacteriol., in press), and R2 (7). Evidence that a toluene-degrading pathway could have a chromosomal location comes from the existence of *P. putida* PAM1, a strain which has the same origin as strain PAW1 and is phenotypically Tol$^+$. However, it harbors a plasmid identical to pWWO-8, which can be expelled from the cell without the loss of the Tol$^+$ function (7).

(A preliminary report of some of these results was presented at a symposium in Santo Domingo in January 1981 [3].)

We thank Philip Lehrbach and John Ward for useful discussions.

We thank the Medical Research Council for support.

LITERATURE CITED

1. Bayley, S. A., C. J. Duggleby, M. J. Worsey, P. A. Williams, K. G. Hardy, and P. Broda. 1977. Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. 154:203–204.
2. Bolivar, F., R. L. Rodriguez, P. J. Green, H. C. Bethach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles, II. A multiple cloning system. Gene 2:75–93.
3. Broda, P., R. Downing, P. Lehrbach, I. McGregor, and P. Meulien. 1981. Degradative plasmids: TOL and beyond, p. 511–517. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Publishing Corp. New York.
4. Dhaese, P., H. DeGreve, H. Decraemer, J. Schell, and M. Van Montagu. 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmid of *Agrobacterium tumefaciens*. Nucleic Acids Res. 7:1836–1849.
5. Downing, R. G., and P. Broda. 1979. A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. Mol. Gen. Genet. 177:189–191.
6. Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda. 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. 130:1274–1280.
7. Jeenes, D. J., and P. A. Williams. 1982. Excision and integration of the degradative pathway genes from the TOL plasmid pWW0. J. Bacteriol. 150:188–194.
8. Meulien, P., R. G. Downing, and P. Broda. 1981. Excision of the 40kb segment of the TOL plasmid pWW0 from *Pseudomonas putida* mt-2 involves direct repeats. Mol. Gen. Genet. 184:97–101.
9. Nakazawa, T., S. Inouye, and A. Nakazawa. 1980. Physical and functional mapping of RP4-TOL plasmid recombinants: analysis of insertion and deletion mutants. J. Bacteriol. 144:223–231.
10. Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. 113:237–245.
11. Thuring, R. W. J., J. P. M. Sanders, and P. Borst. 1975. A freeze squeeze method for recovering long DNA from agarose gels. Anal. Biochem. 66:213–222.
12. Williams, P. A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida (arvilla)* mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. 120:416–423.
13. Worsey, M. J., and P. A. Williams. 1975. Metabolism of toluene and xylenes by *Pseudomonas putida (arvilla)* mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. 124:7–13.

PLASMID 9, 000–000 (1983)

Characterization by Molecular Cloning of Insertion Mutants in TOL Catabolic Functions

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Received August 2, 1982; revised Dec. 2, 1982

A physical and genetic map of the Tol catabolic region of pWWO (TOL) was obtained by restriction endonuclease analysis of several DNA insertion mutants (*xylA*, *xylA xylS*, *xylS*, and *xylR*) of R plasmid–TOL derivatives. In two cases, the inserted DNA was shown from restriction, DNA hybridization, or heteroduplex analysis of cloned *Hin*d III fragments to originate from within pWWO fragment *Hin*d III–E. The effect of these DNA insertions on Tol catabolic activity and on structural alterations to the TOL plasmid is discussed.

Pseudomonas putida mt-2 possesses a degradative plasmid (TOL, pWWO) which codes for a suite of inducible enzymes responsible for the catabolism of aromatic hydrocarbons (e.g., toluene, *m*-, and *p*-xylenes) to central metabolites via the *meta*-cleavage pathway (Worsey and Williams, 1975) (see Fig. 1). A model has been proposed for the control of the synthesis of these enzymes, based on the properties of several *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced mutants of pWWO (Worsey *et al.*, 1978) and from the analysis of partial diploids, using mutants of pWWO and an RP4–TOL recombinant (Franklin and Williams, 1980). In this model, the genes of the degradative pathway, present on pWWO and the RP4-TOL derivatives, are organized into two regulatory units, *xylABC* and *xylDEGF*. These units are under the control of two positive regulator genes *xylR* and *xylS*. Support for this model has recently been obtained from the analysis of several deletion mutants of an RP4–TOL derivative (Nakazawa *et al.*, 1980) and from the modes of expression of TOL catabolic enzymes on cloned restriction fragments of TOL DNA in *Escherichia coli* (Inouye *et al.*, 1981a,b) and *Pseudomonas* (Franklin *et al.*, 1981).

In this paper, we describe the analysis of several spontaneously occurring insertion mutants in two R plasmid–TOL derivatives. In each of the isolates studied, a hitherto unknown 3.4-kb insertion of foreign DNA into TOL DNA accounts for the mutant phenotype. By reference to the restriction map of TOL (Downing and Broda, 1979; Lehrbach *et al.*, 1982) and by cloning the *Hin*dIII-derived fragments of TOL containing the inserted DNA, we have constructed a map of the structural and regulatory functions of TOL. These results confirm those of others (Nakazawa *et al.*, 1980; Inouye *et al.*, 1981a,b; Franklin *et al.*, 1981) and provide additional information on the organization and regulation of TOL catabolic functions.

In addition, we have used Southern hybridization and heteroduplex analysis to identify the origin of the 3.4-kb DNA insert present in two of these strains.

MATERIALS AND METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media and culture conditions. Luria broth was used as the complete medium and M9 as the minimal medium. Cells were grown on

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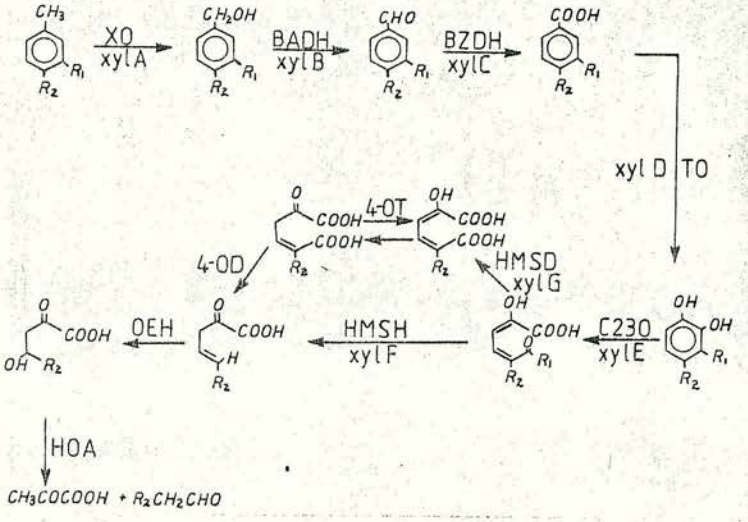

FIG. 1. The degradative pathway encoded by the TOL plasmid. Enzyme abbreviations: XO, xylene oxidase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate oxidase; C230, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; 4-OT, 4-oxalocrotonate tautomerase; 4-OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. Compounds: R1, H; R2, H; toluene. R1, CH_3, R2, H; *m*-xylene. R1, H, R2, CH_3; *p*-xylene. *xylA* to *xylG*, genes for the degradative pathway enzymes.

m-xylene or toluene in liquid or solid media as previously described (Worsey and Williams, 1975). *m*-Toluate was used at a final concentration of 5 mM in M9 minimal medium.

Purification of plasmid DNA and restriction endonuclease analysis. Plasmid DNA from *P. putida* was isolated by a procedure based on the method by Hansen and Olsen (1978). pBR322 and its derivatives were prepared by the cleared lysate method (Guerry *et al.*, 1973). The method of Birnboim and Doly (1979) was used for screening recombinant plasmid DNA species.

Restriction enzymes *Eco*RI (Boehringer–Mannheim), *Hin*dIII (Bethesda Research Lab.), and *Xho*I (Bethesda Research Lab.) were used as specified by the manufacturers. Other enzymes (*Pst*I, *Sal*I, *Pvu*II, *Bam*HI, and *Bgl*II) were prepared in this laboratory by L. Wallace and J. Ward and used as specified in the Bethesda Research Laboratory catalogue.

Digested DNA was analyzed by electrophoresis in 0.5 or 1% agarose gels with a horizontal gel apparatus. Bacteriophage lambda DNA digested with *Hin*dIII provided the DNA size markers.

Ligation and transformation. Treatment of endonuclease-restricted DNA with bacterial alkaline phosphatase and T4 ligase was carried out as specified by the manufacturers (Bethesda Research Lab). Ligation mixtures were used directly to transform $CaCl_2$-treated cells of *E. coli* K-12 strain ED8654. Transformants were selected on nutrient agar containing ampicillin (100 μg ml^{-1}) and then replicated onto nutrient agar containing tetracycline (15 μg ml^{-1}) to distinguish Ap^rTc^s [2] clones. To detect cloned fragments expressing toluate oxidase and catechol 2,3-dioxygenase activities, the transformant mixture was plated on nutrient

[2] Abbreviations used: Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; SSC, sodium chloride, 0.15 M, and sodium citrate, 0.015 M; SDS, sodium dodecyl sulfate; C23O, catechol 2,3-oxygenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; 4-OD, 4-oxalocrotonate decarboxylase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; XO, xylene oxidase.

TABLE 1

BACTERIAL STRAINS

Strain/plasmid	Strain and plasmid characteristics[a]	Source or reference
E. coli K-12		
ED8654	*met*⁻	Borck *et al.*, 1976
ED8654(pBR322)	*met*⁻/pBR322(Ap^{r}Tc^r)	This study
ED8654(pED3306)	*met*⁻/pED3306(Ap^r; contains *Hin*dIII fragment HD of pWWO)	Meulien *et al.*, 1981
ED8654(pED3310)	*met*⁻/pED3310(Ap^r; contains *Hin*dIII fragment HE of pWWO)	This study
ED8654(pMT054)	*met*⁻/pMT054(Ap^r; contains *Hin*dIII fragment HD of pMT051)	This study
ED8654(pMT055)	*met*⁻/pMT055(Ap^r; contains *Hin*dIII fragment HD of pWWO-2023)	This study
ED8654(pMT056)	*met*⁻/pMT056(Ap^r; contains a *Sal* subfragment of pMT054)	This study
ED8654(pMT057)	*met*⁻/pMT057(Ap^r; contains *Hin*dIII fragment HA of pWWO)	This study
ED8654(pMT058)	*met*⁻/pMT058(Ap^r; contains *Hin*dIII fragment HA of pMT053)	This study
Pseudomonas putida		
PaW1	prototrophic/pWWO(Tol$^+$)	Williams and Murray, 1974
PaW153	*trp*⁻*str*^{r}/pED3300(Cb^{r}Kn^{r}Tc^{s}Tol$^+$)	Meulien *et al.*, 1981
MT051	*trp*⁻*str*^{r}/pMT051(Cb^{r}Kn^{r}Tc^{s}Tol⁻[toluene⁻xylene⁻toluate$^+$]	This study
MT052	*trp*⁻*str*^{r}/pMT052(Cb^{r}Kn^{r}Tc^{s}Tol⁻[toluene⁻xylene⁻toluate⁻]	This study
MT053	*trp*⁻*str*^{r}/pMT053(Cb^{r}Kn^{r}Tc^{s}Tol⁻[toluene$^+$xylene⁻toluate⁻]	This study
PRSB23	*trp*⁻*str*^{r}/pWWO-2023(Cb^{r}Kn^{r}Sm^{r}Su^{r}Tol⁻ [toluene⁻xylene⁻toluate$^+$])	Jeenes and Williams, 1982

[a] Abbreviations used: Antibiotic resistances: Cb, carbenicillin; Ap, ampicillin; Tc, tetracycline; Kn, kanamycin, Sm or str, streptomycin, and Su, sulphonamide. Tol, degradation of aromatic hydrocarbons (toluene, *p*- and *m*-xylene, and *m*-toluate).

agar containing carbencillin (500 μg ml^{-1}) and sodium benzoate (800 μg ml^{-1}) and incubated at 30°C for 2 days. Yellow colonies indicative of the formation of 2-hydroxymuconic semialdehyde were purified.

Transfer of DNA to nitrocellulose filters. DNA restriction fragments separated by agarose gel electrophoresis were transferred to nitrocellulose filters (Schleicher and Schuell BA85) by the method of Southern (1975).

Preparation of ^{32}P-labeled plasmid DNA and DNA/DNA hybridization conditions. Plasmid DNA (approx. 1 μg) was ^{32}P-labeled by nick translation essentially as described by Rigby *et al.* (1977) using [^{32}P]dATP as the labeled precursor. After 4 h incubation, the unreacted triphosphates were removed by passing the mixture through a Sephadex G-50 column.

Nitrocellulose filters, containing the denatured DNA fragments, were wetted with 2×

TABLE 2

GROWTH CHARACTERISTICS OF STRAINS OF *P. putida* CARRYING R PLASMID–TOL DERIVATIVES

Growth substrate[b]	Growth of strains[a] (suggested mutant type)				
	PaW153 RP4–TOL (wild type)	MT051 RP4–TOL⁻ (*xylA*)	MT052 RP4–TOL⁻ (*xylA xylS*)	MT053 RP4–TOL⁻ (*xylS*)	PRSB23 R2–TOL⁻ (*xylR*)
Toluene	+	−	−	+	−
m-Methylbenzyl alcohol	+	±	−	−	±
m-Xylene	+	−	−	−	−
p-Xylene	+	−	−	−	−
p-Ethyl toluene	+	−	−	*	−
1,2,4-Methyl benzene	+	−	−	−	−
Toluate	+	+	*	*	+
Benzoate	+	+	+	+	+

[a] +, good growth; ±, slow growth; −, no growth; *, accumulation of catechol (or methylcatechol) in the plate medium (brown color).

[b] Growth tests were conducted on minimal agar plates with benzoate and toluate (10 mM) incorporated into the medium, while the hydrocarbons and alcohol were supplied in the vapor form as described by Kunz and Chapman (1981a).

SSC, and 50% formamide for 10 min at 37°C in confined plastic bags. Excess fluid was then removed. ^{32}P-labeled probe DNA, denatured by boiling for 10 min, was added to the filters. The plastic bags were then sealed and hybridizations were carried out at 37°C for 16 h.

After hybridization, the filters were washed twice in 2× SSC, 0.1% SDS at 37°C, then twice in 2× SSC. For autoradiography, dried filters were exposed to sensitized photographic film next to an intensification screen at −70°C.

Heteroduplex analysis. Preparation of DNA molecules was carried out by the method of Davis *et al.*, 1971. Double-stranded and single-stranded molecules of M13 (6.23 kb) and pAT153 (3.6 kb) were added to provide DNA length standards.

Cell-free extracts and enzyme assays. Preparation of cell extracts were as previously described (Jeenes *et al.*, 1982). The following enzymes were assayed according to published procedures: catechol 2,3-oxygenase (C23O), 2-hydroxymuconic semialdehyde hydrolase (HMSH); 2-hydroxymuconic semialdehyde dehydrogenase (HMSD); 4-oxalocrotonate decarboxylase (4-OD) (Sala-Trepat and Evans, 1971); benzyl alcohol dehydrogenase (BADH), and benzaldehyde dehydrogenase (BZDH) (Jeenes *et al.*, 1982). XO was assayed indirectly in whole cells. The stimulation of uptake of O_2 by the addition of *m*-xylene was measured in an oxygen electrode.

Containment conditions. Experiments involving recombinant DNA generated *in vitro* were carried out under category zero containment conditions as specified by GMAG.

RESULTS

Isolation, Comparative Growth Studies, and Enzyme Assays for R Plasmid–TOL Derivatives

The parent RP4–TOL plasmid (pED3300) used in this study has recently been described (Meulien *et al.*, 1981; Lehrbach *et al.*, 1982); pED3300 contains a 69-kb segment of TOL DNA which specified the toluene-degrading enzymes in the same way as the wild-type TOL plasmid pWWO, from which it was derived. PaW153 (pED3300) is able to grow on a variety of aromatic hydrocarbons (Table 2), a property shared with *P. putida* mt-2 and HS1 (Kunz and Chapman 1981a,b). MT051 was isolated as a spontaneous mutant of

TABLE 3

ACTIVITIES OF PLASMID-CODED ENZYMES IN CELL EXTRACTS OF *Pseudomonas* STRAINS

Strain	Growth substrate[a]	XO	Activities[b] BADH	BZDH	C230	HMSD	HMSH	4-OD
PaW1	Acetate	<0.5	7	7	250	7	30	45
	Acetate/*m*-xylene	4.8	380	660	10,500	250	950	—[c]
	Acetate/*m*-toluate	<0.5	—	—	3,600	185	570	500
MT051	Acetate	<0.5	7	8	290	7	28	—
	Acetate/*m*-xylene	0.7	7	655	7,000	120	400	—
	Acetate/*m*-toluate	<0.5	8	47	4,400	138	627	—
MT052	Acetate	—	—	—	76	3	14	8
	Acetate/*m*-xylene	—	—	—	68	<1	4	—
	Acetate/*m*-toluate	—	—	—	42	2	6	—
MT053	Acetate	—	—	—	38	1	5	18
	Acetate/*m*-xylene	—	—	—	24	1	5	35
	Acetate/*m*-toluate	—	—	—	49	1	9	32

[a] Cells were prepared as previously described (Jeenes *et al.*, 1982).

[b] XO was measured by O_2 uptake of whole cells as described by Jeenes *et al.*, 1982, and assayed for XO by addition of *m*-xylene. Values are expressed in microlitres of O_2 per minute per 2 ml of a cell suspension, corrected for endogenous uptake. BADH, B2DH, C230, HMSD, HMSH, and 4-OT were measured in extracts and expressed as milliunits of activity per milligram of protein.

[c] Not determined.

PaW153 (pED3300) that could utilize *m*-toluate for growth but failed to grow on minimal medium supplemented with *m*-xylene or toluene. Following growth of MT051 in liquid medium containing benzoate (10 mM) as sole carbon source, a mutant unable to grow on *m*-toluate was isolated. This strain MT052 retained catechol 2,3-dioxygenase activity. This was detected by spraying colonies of MT052 with catechol, which is converted enzymatically to a yellow product 2-hydroxymuconic semialdehyde. Revertants of MT052 selected for growth on minimal medium supplemented with toluene but unable to grow on xylene- or toluate-supplemented minimal medium were also obtained. One such isolate, MT053, was retained for further study. The growth characteristics of PaW153 and its derivatives are summarized in Table 2. Those of MT051, MT052, and MT053 suggest that the initial (*xylABC*) and *meta*-cleavage (*xylDEGF*) parts of the pathway are either individually affected (in the case of MT051) or both affected (MT052 and MT053). The accumulation of catechols by MT053 on medium containing *p*-ethyl toluene or *m*-toluate suggests that these two compounds are only partially degraded in this strain.

Measurements of oxidation rates and enzyme assays (Table 3) following growth on various aromatic substrates suggest that MT051 is a *xylA* mutant (defective in xylene oxidase), MT053 is a *xylS* mutant (defective in the regulation of the *meta*-cleavage pathway, and MT052 is a double mutant (*xylA xylS*).

Also included in this study of catabolic mutants was a recently described *xylR* mutant (strain PRSB23) altered in the regulation of the TOL catabolic pathway. PRSB23 contains a plasmid pWWO-2023 consisting of a 56-kb region of TOL DNA and all of the R plasmid R2 (Jeenes *et al.*, 1982; Jeenes and Williams, 1982). PRSB23 is similar to MT051 in failing to grow on toluene or related compounds while showing normal growth on *m*-toluate (Table 2). However data on the non-induced and induced levels of TOL enzymes

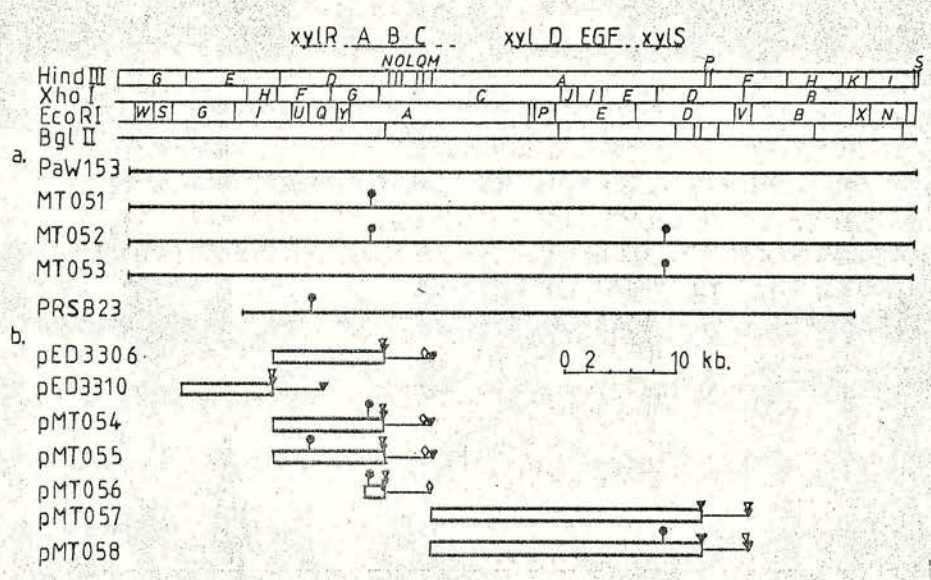

FIG. 2. A restriction map (*Hin*dIII, *Xho*I, *Eco*RI, and *Bgl*II) of TOL (pWWO) showing the region of TOL DNA included in the various R plasmid-TOL hybrids analyzed. The locations of *xylE, xylF*, and *xylG* are taken from Inouye *et al.*, 1981a; *xylB* from Inouye *et al.*, 1981b. *Hin*dIII, *Xho*I, and *Eco*RI fragments are alphabetically labeled according to sizes from the complete TOL (pWWO) map (Lehrbach *et al.*, 1982; Ward and Lehrbach, unpublished). (a) The boundaries of TOL DNA are indicated by a solid line for each R plasmid–TOL hybrid. DNA insertions (▼) are marked within this region. (b) pBR322 recombinant plasmids carrying a particular TOL *Hin*dIII fragment are shown. Symbols for restriction sites are (not all shown): ▽, *Hin*dIII; ▽, *Eco*RI; ◇, *Sal*I.

in these strains distinguishes them; on induction with *m*-xylene MT051 (*xylA*) shows nearly wild-type levels of expression of the *xylDEGF* function (Table 3), whereas PRSB23 (mutant in *xylR*) is noninducible for these functions (Jeenes *et al.*, 1982).

Restriction Endonuclease Analysis of R Plasmid–TOL Derivatives

Initial interest in these mutant plasmids was stimulated by the observation that the mutant phenotype was associated with the insertion of 3.4 kb of foreign DNA into the TOL region. They therefore provided the opportunity of precisely mapping the inactivated TOL structural and regulatory genes.

Figure 2 shows the restriction maps (*Hin*dIII, *Xho*I, *Eco*RI, and *Bgl*II) of the TOL segments of pED3300, pMT051, pMT052, pMT053, and pWWO-2023, together with the positions of the inserts. The *xylA* mutant (MT051) carries inserted DNA in the *Hin*dIII-D fragment. The *xylA xylS* double mutant (MT052) still has this insert and also carries a similar sized insert in *Hin*dIII-A. The revertant strain, MT053, has lost the inserted DNA in *Hin*dIII-D but retains the insert in *Hin*dIII-A.

Restriction of pWWO-2023 DNA using *Hin*dIII and *Xho*I also reveal a 3.4-kb DNA insertion in pWWO fragment *Hin*dIII-D. It was noted that in no case was the inserted DNA itself cut by *Hin*dIII.

To map the various insertions in more detail, the respective *Hin*dIII fragments carrying the insertion were cloned into the *E. coli* vector plasmid pBR322 (Fig. 2). Restriction digests of these recombinant plasmids (pMT054 for pMT051, pMT055 for pWWO-2023, and pMT058 for pMT053) were compared to similar digests of the cloned *Hin*dIII fragments from pWWO (i.e., pED3306 and pMT057). As shown by the comparison between pMT055 and pED3306 (Fig. 3), the insertion is not cut with any of the restriction enzymes tested (*Eco*RI, *Sal*, *Pst*I, and *Pvu*II), so that in each case the size of a single sub fragment

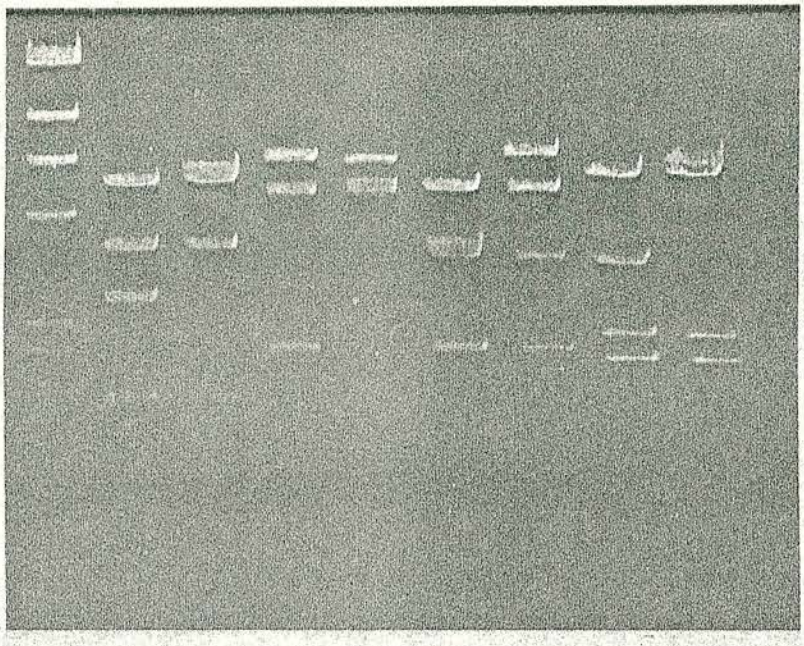

FIG. 3. Agarose gel electrophoresis analysis of pED3306 (b, d, f, h) and pMT055 (c, d, g, i) digested with *Eco*RI (b, c), *Pst*I (d, e), *Pvu*II (f, g), and *Sal*I (h, i). λ DNA (a) digested with *Hin*dIII was used as a size marker (kb).

of *Hin*dIII-D is increased by 3.4 kb. A similar analysis was carried out with pMT054 and pED3306 and the results are summarized in Fig. 4. The DNA insertion in pMT051 is located in a 1.0-kb region of *Hin*dIII-D bounded by an *Xho*I site (the right hand end of *Xho*I-G) and a *Sal*I site (within the *Xho*I-G fragment). The DNA insertion in pWWO-2023 can be mapped to a 1.6-kb region of *Hin*dIII-D bounded by a *Xho*I site (the right hand end of *Xho*I-F) and a *Pvu*II site (internal to *Xho*I-F). It should be noted that *Xho*I-F represents one end of a 40-kb segment of pWWO DNA bounded by 1.4-kb direct repeats (Meulien *et al.*, 1981). From the mapping data for pMT055, the 1.6-kb region of *Xho*I-F in which the insertion occurs overlaps by 0.7 kb with the repeated region; whether the insertion is within the 1.4-kb repeat or in the adjacent 0.9 kb has not been determined. The 1.4-kb direct repeat has been shown to be involved in the generation of specific excisions (Meulien *et al.*, 1981); whether there is a functional relationship between the repeat and the inac-

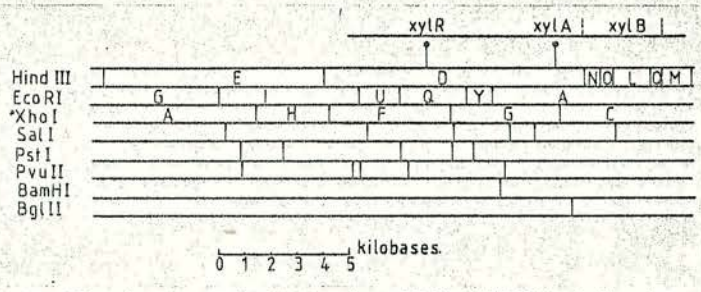

FIG. 4. Physical and functional map of a 24-kb region of TOL(pWWO) including the positions of genes for xylene oxidase (*xylA*), benzyl alcohol dehydrogenase (*xylB*), and a control gene (*xylR*). (a) The positions of DNA insertions (↑) used to define these loci (*xylA* and *xylR*) are shown. The location of *xylB* is taken from Inouye *et al.*, 1981a.

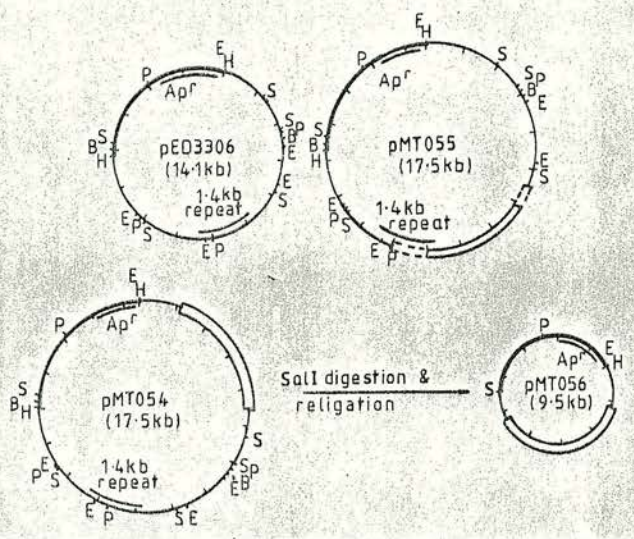

FIG. 5. Physical maps of plasmids pED3306, pMT054, pMT055, and pMT056. Restriction endonuclease cleavage sites for *Eco*RI (E), *Bam*HI (B), *Pvu*II (P), and *Sal*I (S) are indicated. The area indicated: ▩, pBR322 DNA; □, cloned insertion DNA (3.4 kb); and —, cloned *Hin*dIII-D TOL DNA. The ampicillin resistance gene (Ap^r) of pBR322 and a 1.4-kb region homologous with *Hin*dIII-F TOL DNA are shown (see text for explanation). Scale divisions (inside circle) 1 kb.

tivated gene(s) defined in pWWO-2023 is not clear.

A similar analysis was carried out with the insertion in *Hin*dIII-A from pMT053. The recombinant plasmid carrying this fragment (pMT058) was compared by restriction analysis (*Hin*dIII, *Xho*I, *Eco*RI, and *Bgl*II) with the hybrid plasmid carrying the *Hin*dIII-A fragment (pMT057). The results are summarized in Fig. 2. The 3.4-kb DNA insertion in *Hin*dIII-A can be mapped to a 1.8-kb region bounded by a *Xho*I site (the left-hand end of *Xho*I-D) and a *Bgl*II site (within *Xho*I-D).

Origin and Size of the DNA Insertion

Restriction analyses of pMT054 and pMT055 suggested that the inserted DNA present in these cloned derivatives of pMT051 and pWWO-2023 may be related. In each case, the inserted DNA present in the *Hin*dIII-D fragment was not cut by the eight restriction enzymes tested and the inserted DNA was approximately the same size in each case (3.4 kb). To test these relationships in more detail, Southern hybridization analysis was used. In order to obtain a recombinant plasmid with a subfragment of *Hin*dIII-D containing the insertion, pMT054 DNA was digested with *Sal*I, the resulting restriction fragments were religated, and Ap^r transformants selected. An Ap^r clone was purified and shown to harbor a plasmid pMT056 containing a single 8.5-kb *Sal*I DNA fragment originating from pMT054 (Fig. 2). Plasmids pMT056 and pMT055 were selected for hybridization studies; their physical maps are illustrated in Fig. 5.

Radioactive ^{32}P-labeled plasmid probes of pMT056 and pMT055 were used to investigate the sequence homologies between these plasmids and the parental TOL plasmid pWWO.

The hybridization pattern of digests of pMT055 (*Pvu*II) (lanes a and e), pED3306 (*Eco*RI) (lanes b and f), pMT054 (*Sal*I) (lanes c and g), and pWWO (*Hin*dIII) (lanes d and h), using pMT055 or pMT056 plasmid DNA as probes, are shown in Figs. 6A and B, respectively.

As can be seen in Fig. 6A, pMT055 DNA hybridized to all the digestion fragments of pMT055 (lane a), pED3306 (lane b), and pMT054 (lane c) since it contains all the DNA sequences present in these plasmids (see Fig.

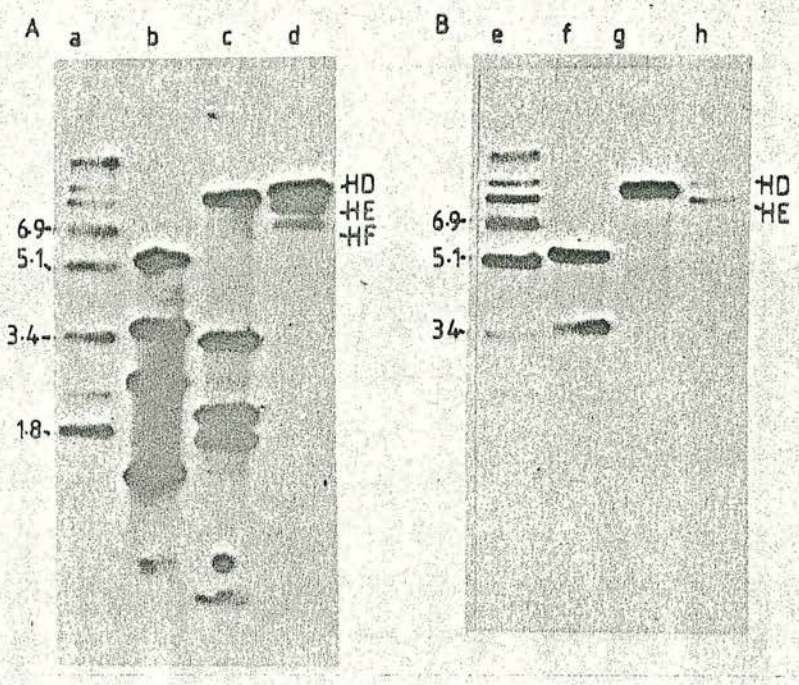

FIG. 6. Southern blot hybridization of ^{32}P-labeled recombinant DNA (A) pMT055 and (B) pMT056 to *Pvu*II-digested pMT055 (a, e), *Eco*RI-digested pED3306 (b, f), *Sal*I-digested pMT054 (c, g), and *Hin*dIII-digested pWWO (d, h). Higher molecular size PvuII fragments in lanes a and e represent hybridization to partial digestion products.

5). pMT055 DNA also hybridizes to three *Hin*dIII pWWO fragments, *Hin*dIII-D (HD) as expected, *Hin*dIII-F (HF) since HD shares 1.4 kb of homology with *Hin*dIII-F (Meulien *et al.*, 1981), and a 8.4-kb fragment *Hin*dIII-E (HE). As previous hybridization experiments (data not shown) with pED3306 DNA showed homology with only *Hin*dIII-D and *Hin*dIII-F, this result with pMT055 DNA provided evidence that the insertion (3.4 kb) present in pMT055 DNA was responsible for the homology with the *Hin*dIII-E fragment of pWWO DNA.

pMT056 probe DNA (Fig. 6B) hybridizes to selected fragments of pED3306 (lane f) and pMT054 (lane g) since it is a deleted derivative of pMT054 which contains only 2 kb of the *Hin*dIII-D pWWO fragment and 3.4 kb of the insertion DNA. As can also be seen in Fig. 6B, pMT056 DNA hybridizes to three *Pvu*II-generated fragments in pMT055 DNA with lengths of 6.9, 5.1, and 3.4 kb (higher molecular size *Pvu*II fragments in lanes a and e represent hybridization to partial digestion products). The restriction map of pMT055 DNA (Fig. 5) reveals that homology of pMT056 DNA with the 5.1- and 3.4-kb fragments can be explained by common pBR322 DNA sequences. The 6.9-kb *Pvu*II segment represents an internal 3.5-kb *Pvu*II fragment of *Hin*dIII-D in which the inserted DNA is present in pMT055 (Fig. 3, lane g). Thus, sequence homology between the 6.9-kb *Pvu*II fragment and pMT056, in which the original 3.5-kb *Pvu*II fragment has been deleted, can only be explained by the common 3.4-kb insertion present in both pMT056 and pMT055. pMT056 DNA hybridizes to *Hin*dIII-D (HD) and *Hin*dIII-E (HE) fragments of pWWO DNA.

To summarize, Southern hybridization analysis provides strong evidence that the DNA insertions responsible for the inactivation of different TOL catabolic functions in MT051 and PRSB23 are identical and originate from a region of pWWO DNA (*Hin*dIII-E restriction fragment) distinct from the inactivated gene(s).

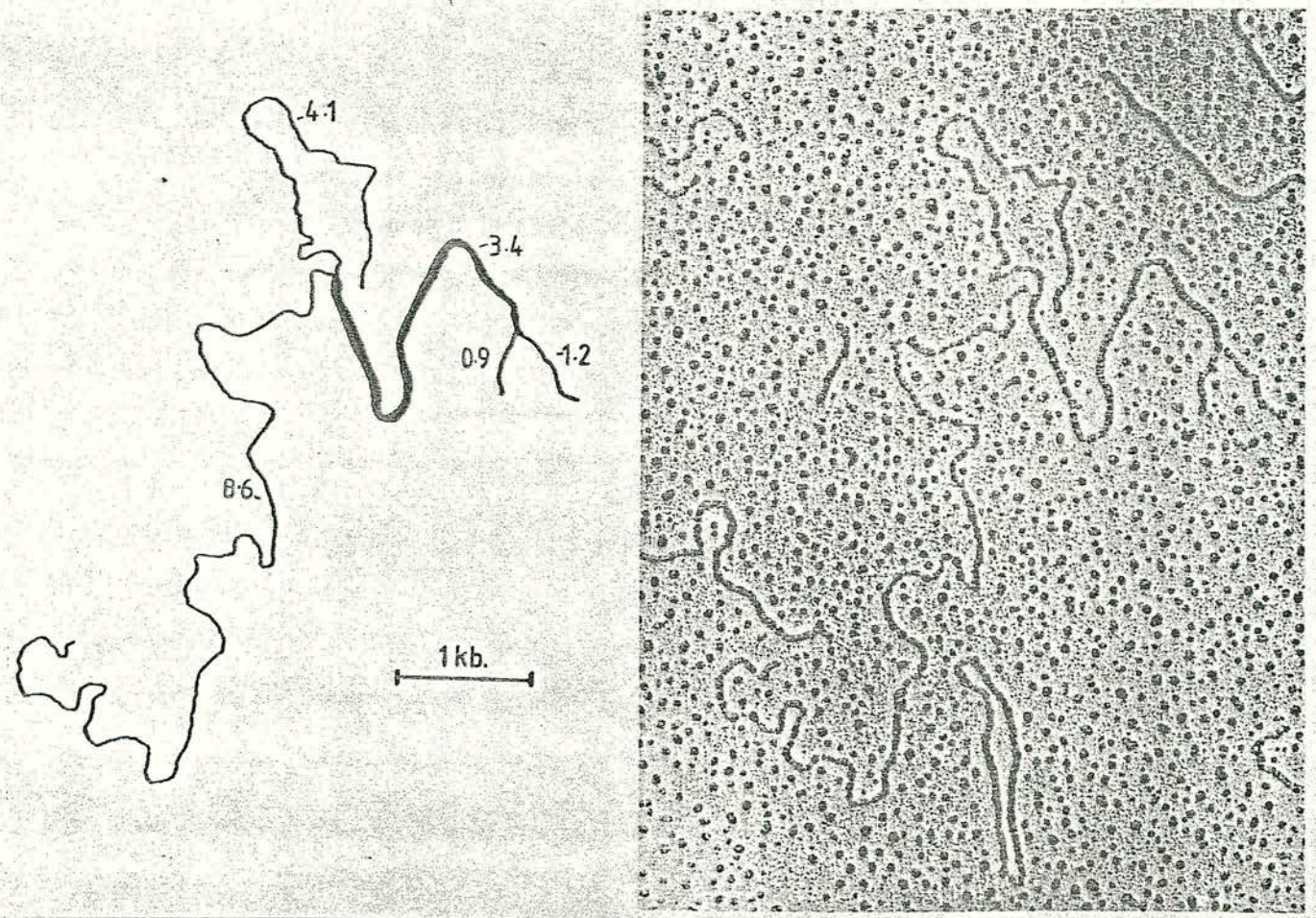

FIG. 7. Electron micrograph and tracing of reannealed single-stranded *Hind*III generated fragments of pED3310 and pMT054. The calculated DNA lengths are in kilobases. Molecular weight standards were M13 (6.23 kb) and pAT153 (3.66 kb).

An accurate determination of the size and location of the 3.4-kb element within *Hin*dIII-E was obtained by electron microscopy. Heteroduplexes were formed between *Hin*dIII-generated fragments of pED3310 and pMT054, and 15 heteroduplex molecules of the configuration shown in Fig. 7 were photographed and traced. As shown in Fig. 7, a 3.4-kb double-stranded region is present at 0.8 kb from the end of *Hin*dIII-E. The location of the 3.4-kb element within *Hin*dIII-E is in good agreement with the expected location from restriction analysis. This region at the left hand end of *Hin*dIII-E (Fig. 4), as is the case with the insertion DNA, is devoid of restriction sites for *Hin*dIII, *Eco*RI, *Sal*I, *Pvu*II, *Xho*I, *Pst*I, *Bam*HI, and *Bgl*II.

The extent of homology between the DNA insertion in *Hin*dIII-A (from pMT051 and pWWO-2023) has not been determined; however, they appear similar on the basis of size (3.4 kb) and the absence of a number of restriction sites.

DISCUSSION

In this study, we have analyzed four mutant TOL derivatives (*xylA, xylA xylS, xylS,* and *xylR*) in which a DNA insertion inactivates the particular TOL function. By molecular cloning of individual TOL *Hin*dIII-generated fragments, a restriction map of particular regions of the TOL plasmid (pWWO) was obtained and the insertions mapped.

Two mutant TOL plasmid derivatives pMT051 and pWWO-2023, containing insertions in the *Hin*dIII-D fragment, do not allow host bacteria (MT051 and PRSB23, respectively) to utilize the hydrocarbons (*m*-xylene or toluene) as carbon source, whereas the host cells can use the corresponding acid metabolite (toluate or benzoate) as carbon source. These properties indicate that the upper pathway structural or control genes are inactivated in these host cells and are on the plasmid *Hin*dIII-D fragment. Oxygen uptake studies and enzyme assays (Table 3) demonstrated that bacteria carrying the pMT051 plasmid did not contain xylene oxidase (*xylA*) and showed significantly reduced levels of BADH (*xylB*). However, detectable levels of BZDH (*xylC*) were found which showed the same induction pattern as PAW1 for the aromatic substrates tested (Table 3). Thus the insertion in pMT051 located 1.2 kb from the end of *Hin*dIII-D (see Fig. 4) is in or near the *xylA* gene and is polar on *xylB* but not *xylC*. These results may indicate the *xylAB* genes form a regulon that is transcribed in the direction *xylA–xylB*, consistent with previous reports (Nakazawa *et al.*, 1980; Franklin *et al.*, 1981) but suggest that *xylC* is under separate regulatory control.

The growth phenotype and enzyme induction pattern of host cells carrying plasmid pWWO-2023 suggested that the insertion located within a 1.6-kb region of the *Hin*dIII-D fragment inactivated a gene (*xylR*) involved in the regulation of the *xylAB(C)* regulon. It is postulated (Worsey *et al.*, 1978; Franklin and Williams, 1980) that hydrocarbon substrates (and alcohols) interact with the *xylR* gene product to induce the synthesis of the upper pathway and *meta*-cleavage enzymes.

The enzyme induction pattern of PRSB23, as well as related derivatives WR211 and PRSB1 (Jeenes *et al.*, 1982; Jeenes and Williams, 1982), are similar to the previously defined *xylR* mutant PaW210 (Worsey *et al.*, 1978) and it was considered likely that PaW210 (pWWO-210) was also mutated in a similar region of the *Hin*dIII-D fragment. However complementation studies in *Pseudomonas* with cloned *Hin*dIII-D and *Xho*I-G fragments (Franklin *et al.*, 1981) showed that they did not restore the ability of PaW210 to grow on *m*-xylene and the authors concluded that the mutation in pWWO-210 was located downstream (i.e., in *Hin*dIII-HA or -HF fragments) of the *xylAB(C)* regulon. Thus, it would appear that more than one gene function is involved in the complex coordinate regulation of the upper and *meta*-cleavage pathways; further studies are necessary to distinguish between these components of the regulatory system.

The analysis of insertion and mutant plas-

mid derivatives from strains MT052 and MT053 allows the localization of a control gene (*xylS*) for the *meta*-cleavage pathway. During this study, the growth characteristics of MT052 and MT053 originally suggested that these strains carried plasmids (pMT052 and pMT053, respectively) that may have been mutated in a structural gene lower down the *meta*-cleavage pathway than the previously characterized *xylE* gene (Nakazawa *et al.*, 1980) or genes for enzymes of the 4-oxalocrotonate branch of the *meta*-cleavage pathway (Fig. 1). Such a mutant derivative, defective either in 2-oxopent-4-enoate hydratase or 4-hydroxy-2-oxovalerate aldolase, would be expected to allow host cells to use toluene as the sole carbon source, since the metabolite benzoate could be metabolized via *ortho* cleavage, whereas other hydrocarbons (*p*- or *m*-xylene) and their derivatives could not be metabolized in this way. However, such a mutant would not be expected to alter the inducibility of earlier *meta*-cleavage enzymes in the presence of the hydrocarbons or acid metabolites. As the enzyme data shows, induction of catechol 2,3-dioxygenase activity by *m*-xylene or *m*-toluate is abolished; this most likely suggests that MT053 (and MT052) is not blocked in a *meta*-cleavage structural gene but in a control gene. The possibility that the DNA insertion in pMT053 may have occurred within a structural gene which has a polar effect on the expression of the regulatory gene *xylS* seems unlikely since the *meta*-cleavage pathway structural genes and the *xylS* gene are thought to be separately transcribed (Inouye *et al.*, 1981b). In addition, these results suggest that induction of *meta*-cleavage enzymes by initial methyl-substituted hydrocarbons requires the cooperative action of both the *xylR* and *xylS* gene products for maximal expression of the TOL structural gene units. These results thus point to a more complex regulatory mechanism than originally outlined (Worsey *et al.*, 1978). Details of a revised model based on the properties of these mutants (and others) will be presented elsewhere.

The insertion in *xylS* locates this gene to a 1.6-kb region of *Xho*I-D within *Hin*dIII-A. This is in good agreement with the 3.0-kb *Pst*I fragment previously defined for the *xylS* gene (Inouye *et al.*, 1981b).

The *xylS* plasmid derivative was isolated following serial growth on benzoate (10 mM) as sole carbon source. Growth under these conditions has previously (Bayley *et al.*, 1977) been shown to lead to TOL$^-$ segregants of PaW1 in which either total loss or specific deletions of pWWO occur (Bayley *et al.*, 1977; Meulien *et al.*, 1981). However, in this study, by selecting TOL$^-$ derivatives which retained catechol 2,3-oxygenase activity, we isolated an insertion derivative. These observations point to the usefulness of using benzoate curing to generate a variety of mutants defective in the *meta*-cleavage pathway.

The properties of the 3.4-kb insertion are similar in some ways to those of transposable genetic elements (Starlinger, 1980). In particular, the element identified in this study can insert into the recipient replicon at several locations, which can lead to insertion inactivation of structural and regulatory genes. Furthermore, by hybridization studies (Lehrbach, unpublished data), we have demonstrated that the insertion found in the RP4–TOL derivative pTN1 described by Nakazawa *et al.*, 1980 is very similar to the DNA insertion in pMT051 and pWWO-2023. Thus, the 3.4-kb element can excise precisely from pTN1 and can cause deletions of nearby DNA segments (Nakazawa *et al.*, 1980). Although the structure of the insertion sequence has not been studied in detail, these properties indicate that the 3.4-kb element is resident on the pWWO plasmid (within the *Hin*dIII-E fragment) and is capable of transposing to other sites on pWWO. The ability of this element to transpose to other replicons, including the host chromosome and its relationship to known IS elements, have not been assessed.

It has been proposed that the chromosome mobilizing ability (*cma*) of the broad host range plasmid R68.45 is due to the formation of a R68.45-chromosome cointegrate during

transposition of the insertion sequence IS21 (Willetts *et al.*, 1981). Such a model may explain the *cma* of the TOL plasmid in *Pseudomonas* (White and Dunn, 1977) if the 3.4-kb sequence is capable of transposing to the host chromosome and thus providing the basis for chromosome/plasmid cointegrate formation, a prerequisite for chromosome transfer (Davidson *et al.*, 1975; Willetts *et al.*, 1981).

The identification of this 3.4-kb insertion sequence may also be of relevance to the pWWO DNA structural alterations observed when pWWO is introduced to the haloaromatic degrading *Pseudomonas* sp. B13(WR1) (Reineke *et al.*, 1980; Jeenes *et al.*, 1982). In these studies, the transfer of the TOL plasmid (pWWO) into WR1 enabled novel strains to be isolated which can utilize various chloro-substituted benzoates as their carbon source (Reineke and Knackmuss, 1979, 1980). The utilization of these substrates was shown to depend upon combined activities of a broad specificity TOL enzyme (*xylD*, toluate oxidase) and WR1 host enzymes. However, in all the novel strains analyzed, no catechol 2,3-dioxygenase from pWWO was detected, so that degradation of chlorocatechols by the *meta*-cleavage pathway, which would produce toxic cleavage products, was avoided. Plasmid DNA isolated from these transconjugant strains (eg. WR211, WR216) (Jeenes *et al.*, 1982) showed that pWWO had undergone major structural alterations including deletions and the addition of one or more (approximately) 3-kb insertions; one of which inactivated catechol 2,3-dioxygenase (*xylE*) (Jeenes *et al.*, 1982).

PRSB23 analyzed in this study is a R2-TOL (*xylR*) derivative from the transconjugant WR211 which carries a 3-kb (approx) insertion in the *xylR* gene (Jeenes *et al.*, 1982). We have established that the 3.4-kb insertion is pWWO DNA, present in the *Hin*dIII-E fragment. It therefore appears feasible that the other 3-kb (approx) insertions found in other transconjugants have a similar origin. The involvement of an insertion sequence could thus explain at least some of the complex rearrangements found for the TOL plasmid in the WR1 genetic background.

ACKNOWLEDGMENTS

We are grateful to John Ward for providing pMT057, and Pam Beattie and Pierre Meulien for help with the electron microscopy that was performed at the Department of Molecular Biology, University of Edinburgh. We thank the Medical Research Council for support to P.B. and the Science and Engineering Research Council for a grant to P. A. Williams that supported D.J.J.

REFERENCES

BAYLEY, S. A., DUGGLEBY, C. J., WORSEY, M. J., WILLIAMS, P. A., HARDY, K. G., AND BRODA, P. (1977). Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. *Mol. Gen. Genet.* **154**, 203–204.

BIRNBOIM, H. C., AND DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acid Res.* **7**, 1513–1523.

BORCK, J., BEGGS, J. D., BRAMMAR, W. J., HOPKINS, A. S., AND MURRAY, N. E. (1976). The construction *in vitro* of transducing derivatives of phage lambda. *Mol. Gen. Genet.* **146**, 199–207.

DAVIDSON, N., DEONIER, R. C., HU, S., AND OHTSUBO, E. (1975). Electron microscope heteroduplex studies of sequence relations among plasmids in *E. coli*. The DNA sequence organisation of F and F-primes and the sequence involved in Hfr formation. *In* "Microbiology 1974" (D. Schlessinger, ed.), pp. 56–65. Amer. Soc. Microbiol., Washington, D. C.

DAVIS, R. W., SIMON, M., AND DAVIDSON, N. (1971). EM heteroduplex methods for mapping regions of base sequence homology in nucleic acids. *In* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 21, pp. 413–428. Academic Press, New York.

DOWNING, R. G., AND BRODA, P. (1979). A cleavage map of the TOL plasmid of *Pseudomonas putida* mt-2. *Mol. Gen. Genet.* **177**, 189–191.

FRANKLIN, F. C. H., AND WILLIAMS, P. A. (1980). Construction of a partial diploid for the degradative pathway encoded by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2: Evidence for the positive nature of the regulation by the *xylR* gene. *Mol. Gen. Genet.* **177**, 321–328.

FRANKLIN, F. C. H., BAGDASARIAN, M., BAGDASARIAN, M. M., AND TIMMIS, K. N. (1981). Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring *meta*-cleavage pathway. *Proc. Nat. Acad. Sci. USA* **78**, 7458–7462.

GUERRY, P., LEBLANC, D. J., AND FALKOW, S. (1973). General method for the isolation of plasmid deoxyribonucleic acid. *J. Bacteriol.* **116**, 1064–1066.

HANSEN, J. B., AND OLSEN, R. H. (1978). Isolation of

large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.* **135,** 227–238.

INOUYE, S., NAKAZAWA, A., AND NAKAZAWA, T. (1981a). Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli*. *J. Bacteriol.* **145,** 1137–1143.

INOUYE, S., NAKAZAWA, A., AND NAKAZAWA, T. (1981b). Molecular cloning of *xylS* gene of the TOL plasmid: Evidence for positive regulation of the *xylDEGF* operon by *xylS*. *J. Bacteriol.* **148,** 413–418.

JEENES, D. J., REINEKE, W., KNACKMUSS, H-J., AND WILLIAMS, P. A. (1982). The TOL plasmid pWWO in constructed halobenzoate degrading *Pseudomonas* strains: Enzyme regulation and DNA structure. *J. Bacteriol.* **150,** 180–187.

JEENES, D. J., AND WILLIAMS, P. A. (1982). Excision and integration of the degradative pathway genes from the TOL plasmid pWWO. *J. Bacteriol.* **150,** 188–194.

KUNZ, D. A., AND CHAPMAN, P. J. (1981a). Catabolism of pseudocumene and 3-ethyl toluene by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for new functions of the TOL (pWWO) plasmid. *J. Bacteriol.* **146,** 179–191.

KUNZ, D. A., AND CHAPMAN, P. J. (1981b). Isolation and characterisation of spontaneously occurring TOL plasmid mutants of *Pseudomonas putida* HS1. *J. Bacteriol.* **146,** 952–964.

LEHRBACH, P. R., WARD, J. M., MEULIEN, P., AND BRODA, P. (1982). Physical mapping of the TOL plasmids pWWO and pND2 and various R plasmid-TOL derivatives from *Pseudomonas*. *J. Bacteriol.*, in press.

MEULIEN, P., DOWNING, R. G., AND BRODA, P. (1981). Excision of the 40kb segment of the TOL plasmid from *P. putida* mt-2 involves direct repeats. *Mol. Gen. Genet.* **184,** 97–101.

NAKAZAWA, T., INOUYE, S., AND NAKAZAWA, A. (1980). Physical and functional mapping of RP4-TOL plasmid recombinants: Analysis of insertion and deletion mutants. *J. Bacteriol.* **144,** 222–231.

REINEKE, W., AND KNACKMUSS, H-J. (1979). Construction of haloaromatics utilizing bacteria. *Nature (London)* **277,** 385–386.

REINEKE, W., AND KNACKMUSS, H-J. (1980). Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. *J. Bacteriol.* **142,** 467–473.

REINEKE, W., JEENES, D. J., WILLIAMS, P. A., AND KNACKMUSS, H-J. (1982). The TOL plasmid pWWO in constructed halobenzoate degrading *Pseudomonas* strains: Prevention of *meta*-cleavage. *J. Bacteriol.* **150,** 195–201.

RIGBY, P. W., DIECKMANN, M., RHODES, C., AND BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. *J. Mol. Biol.* **113,** 237–245.

SALA-TREPAT, J. M., AND EVANS, W. C. (1971). The *meta*-cleavage of catechol by *Azotobacter* species. *Eur. J. Biochem.* **20,** 400–413.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–518.

STARLINGER, P. (1980). IS elements and transposons. *Plasmid* **3,** 241–259.

WHITE, G. P., AND DUNN, N. W. (1977). Apparent fusion of the TOL plasmid with the R91 drug resistance plasmid in *Pseudomonas aeruginosa*. *Aust. J. Biol. Sci.* **30,** 345–355.

WILLETTS, N. S., CROWTHER, C., AND HOLLOWAY, B. W. (1981). The insertion sequence IS21 of R68-45 and the molecular basis for mobilization of the bacterial chromosome. *Plasmid* **6,** 30–52.

WILLIAMS, P. A., AND MURRAY, K. (1974). Metabolism of benzoate and the methyl benzoates by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for the existence of a TOL plasmid. *J. Bacteriol.* **120,** 416–423.

WORSEY, M. J., AND WILLIAMS, P. A. (1975). Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: Evidence for a new function of the TOL plasmid. *J. Bacteriol.* **124,** 7–13.

WORSEY, M. J., FRANKLIN, F. C. H., AND WILLIAMS, P. A. (1978). Regulation of the degradative pathway coded for by the TOL plasmid (pWWO) for *Pseudomonas putida* mt-2. *J. Bacteriol.* **134,** 757–764.

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Molec. gen. Genet. 133, 141—149 (1974)

Plasmid Identification Using Specific Endonucleases

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Received July 10, 1974

Summary. Digestion with specific endonucleases followed by agarose gel electrophoresis yields characteristic fragment patterns from different plasmid DNAs. Since even the very closely related R factors R100-1 and R6, for example, can be distinguished this method provides a powerful test of the identity of two plasmid DNAs.

Introduction

In the study of the epidemiology of R factors there is a need for a method of deciding whether two bacterial isolates carry identical plasmids (Richmond, 1973). Several biological and molecular properties have been used to characterise plasmids. These include drug-resistance markers carried, incompatibility, size of plasmid DNA and DNA-DNA hybridisation (Hedges and Datta, 1972; Chabbert *et al.*, 1972; Grindley *et al.*, 1972, 1973; Grinsted *et al.*, 1972; Guerry and Falkow, 1971). Clearly, however, to demonstrate "identity" techniques giving a higher resolution must be used. Richmond (1973) has advocated heteroduplex analysis (Sharp *et al.*, 1972) for this purpose. We show here that when specific endonucleases break plasmid DNAs into fragments these can be resolved on agarose gels into specific patterns, thus providing a sensitive method of distinguishing between plasmids.

Materials and Methods

Materials

Reagents, enzymes and their sources were as follows: agarose, Koch-Light Laboratories Ltd., Colnbrook, Bucks, England; Dowex 50 W-X 8 20—50 U.S. mesh (H), BDH Chemicals Ltd., Poole, England; ethidium bromide, B grade, Calbiochem, Los Angeles, California; lysozyme, Worthington, Freehold, N.J.; Triton X-100, Sigma Chemical Company, St. Louis, Mo. Endo R. *Eco*RI (in the nomenclature of Smith and Nathans, 1973) was prepared from the *E. coli* K12 derivative 1100 (RY5, R1—19) by the procedure of Yoshimori (1971) and was the generous gift of K. Murray. Endo *Ava*I was prepared from *Anabaena variabilis* (K. Murray, S. G. Hughes, J. S. Brown, S. A. Fleming, unpublished). The endonucleases were titrated against 4 μg F DNA to determine the amount required to bring the reaction to completion, this being judged by there being no further change in the fragment pattern.

The strains used are described in Table 1. Strain ED879 is a derivative of strain X637 (Curtiss and Renshaw, 1969) carrying the F factor of the wild-type K12 strain (strain EMG2 of Clowes and Hayes, 1968).

Isolation of Plasmid DNA

Cells were grown in 1 or 2 l. of L broth to 5×10^8 cells/ml or overnight. The following protocol is for a 2 l. culture. Cells were harvested by centrifugation and resuspended in 48 ml of cold 25% sucrose, 0.05 M tris-HCl pH 8.0. Lysozyme (6.9 ml of freshly prepared solution

Table 1. Bacterial strains and Plasmids

Strain	Chromosomal genotype	Plasmid	Source	Reference to plasmid
ED879	*lacY*	F	P. Broda	see text
W1655	*metB*	FΔ(33–43)	P. Broda	Anthony *et al.* (in press)
ED1979	*trp lac*	R100-1	D. Finnegan	Egawa and Hirota (1962)
ED1980	*trp lac*	R1-19	D. Finnegan	Meynell and Datta (1967)
J62-1(R6)	*pro his trp lac nal*	R6	N. Datta	Watanabe *et al.* (1964)
J53(R6K)	*pro met*	R6K	N. Datta	Kontomichalou *et al.* (1970)
1100(R245)	*thi endI*	R245	N. E. Murray	Bannister (1969)

at 10 mg/ml in 0.25 M tris-HCl pH 8.0) was added; the mixture was shaken for 30″ at 37° C, and then placed on ice. After 5′, 26 ml 0.25 M EDTA (pH 8.0) were added and after a further 5′ the cells were lysed by adding 54 ml of a solution containing 2% Triton X-100, 0.05 M tris-HCl (pH 8.0) and 0.0625 M EDTA. A period of 20′ was allowed for lysis, and then the crude lysate was cleared at 17 k.r.p.m. in a Sorvall SS34 rotor for 15′ at 4° C. The supernatant (ca. 130 ml) was divided into equal volumes and layered over 3 ml saturated CsCl cushions (61.7% (w/w) CsCl) in three Spinco SW25.2 tubes. The tubes were completely filled by layering distilled water over the cleared lysates.

The DNA in the cleared lysate was then pelleted into the CsCl cushion by centrifugation at 23 k.r.p.m. and 15° C in a Spinco SW25.2 rotor for 16—20 h. The lowest 8 ml of each tube was collected and these fractions were pooled. Ethidium bromide solution (10 mg/ml) was added to a final concentration of 500 μg/ml. The density was adjusted to 1.55 g/cc (48.4% (w/w) CsCl) in a total volume of 30 ml with CsCl and TES (0.05 M tris-HCl, 0.05 M NaCl, 0.005 M EDTA, pH 8.0). The DNA was banded at 43 k.r.p.m. and 15° C in a Spinco SW50.1 rotor for 20—24 h (Radloff *et al*, 1967).. The lower, red, band comprising closed circular plasmid DNA molecules was collected by aspiration (19 gauge needle, 1 ml disposable syringe). The plasmid bands were pooled and re-centrifuged in two 5 ml volumes for 16—20 h. The plasmid bands were collected and ethidium bromide was removed by dialysis against 10 g Dowex Na^+ resin in 50 ml buffer (0.8 M NaCl, 0.05 M tris-HCl, 0.01 M EDTA, pH 8.0(Sharp *et al.*, 1972)). The resin had been pre-washed with 2 M HCl followed by 1 M NaOH to convert it to the Na^+ form. The DNA solution was finally dialysed into TE buffer (0.01 M tris-HCl, 0.001 M EDTA, pH 7.2). The DNA concentration was calculated spectrophotometrically assuming that an absorbance at 260 nm of 1.0 corresponds to 50 μg/ml DNA. Yields of between 100 and 300 μg plasmid DNA were obtained.

Phage λ DNA was prepared as described by Kaiser and Hogness (1960).

Endonucleolytic Digestion of Plasmid DNA

Digests were performed in small tubes (12.5 mm × 50 mm) at 37° C for 1.5—2 h. Reaction volumes were usually less than 60 μl, containing 10—50 μl DNA preparation (2—8 μg in TE buffer) and 2 μl of endo R.*Eco*RI or 5 μl of endo *Ava*I and adjusted to 0.1 M tris-HCl pH 7.4 and 0.01 M $MgSO_4$. After incubation 10 μl of electrophoresis buffer containing 50% (v/v) glycerol and 0.004% (w/v) bromophenol blue was mixed with the contents of each tube and the liquid volume was reduced to ca. 20 μl by evaporation in a vacuum desiccator. The samples were then analysed by electrophoresis in 1% agarose gels (Hayward and Smith, 1972).

Agarose Gel Electrophoresis

Electrophoresis was carried out on vertical slab gels. The gels were prepared by refluxing 2.5 g agarose with 250 ml electrophoresis buffer (E buffer, 40 mM Tris-acetate, 20 mM sodium acetate, 5 mM EDTA, pH 8.2)(Hayward and Smith, 1972) until dissolved. The solution was

allowed to cool to ca. 50° C and ethidium bromide solution was added to 0.5 μg/ml (Sharp) Sugden and Sambrook, 1973). The gel was cast in a space (0.4 cm × 17 cm × 40 cm) between two glass plates, one of which had been pretreated by coating with 0.1% (w/v) agarose solution which was dried onto the glass at 60° C to prevent gel slippage. The plates were separated by perspex strips at the sides and a perspex slot-former, giving 1 cm gel slots at the bottom. All joints were coated with paraffin wax and held together by spring clips. When set the gel was inverted into the running position, the slotformer removed and the slots filled with E buffer. Samples were layered into the gel slots and electrical contact between the upper buffer reservoir and the buffer over the gel was effected by six layers of Whatman 3 MM aper soaked in E buffer; the bottom of the gel dipped into the lower buffer reservoir. All of the buffer contained 0.5 μg/ml ethidium bromide. Gels were run at constant current of 40 mA for 15—20 h. Until the blue tracking dye was almost at the bottom. Then one glass plate was removed and the gel viewed under short wave ultraviolet light (ChromatoVue, UV Products Inc., San Gabriel, California, USA). The DNA bands fluoresce orange against a dark background and were photographed through a 4 × red filter on Ilford FP4 film.

Results

Comparison of F, R6K and R245

When supercoiled plasmid DNA prepared as described in Materials and Methods is electrophoresed in 1% agarose gels the majority of the DNA migrates as a single band. The remainder of the DNA migrates as a minor band ahead of, and well resolved from, the main band. By examining the behaviour upon electrophoresis of nicked and linear plasmid DNA (from the less dense band in the final dye-CsCl gradient) it was established that the minor component of the supercoiled DNA preparations consists mainly of open circular molecules which presumably arise either by nicking during collection or from contamination with light band material.

Digestion of circular plasmid DNA with specific endonucleases generates a family of linear duplex DNA fragments which can be fractionated according to size in agarose gels (Sharp, Sugden and Sambrook, 1973; Cohen *et al.*, 1973). Fig. 1 shows that each plasmid gives a characteristic band pattern. The enzymes used were the restriction enzyme endo R.*Eco*RI (Yoshimori, 1971) and an endonuclease recently isolated from *Anabaena variabilis*, endo *Ava*I (K. Murray, S. G. Hughes, J. S. Brown and S. A. Fleming, unpublished). In this plate the contrast is reversed so that the fluorescent DNA bands appear dark on a light background. Fragments of phage λ DNA produced by endo R.*Eco*RI, which have been measured by Allet *et al.* (1973), are included in this plate as molecular weight markers.

The three plasmids F, R6K and R245 are in different compatibility groups (Hedges and Datta, 1972; Hedges *et al.*, 1973; Datta and Hedges, 1971) and thus are probably unrelated. The molecular weight of the sex factor F is 62.5×10^6 (Sharp *et al.*, 1972); R6K and R245 have molecular weights of about 26×10^6 (Kontomichalou *et al.*, 1970) and 25×10^6 (R. Thompson, unpublished data) respectively. Table 2 gives molecular weight estimates for the DNA bands in Fig. 1; in each the sum of the fragment molecular weights is in reasonable agreement with the molecular weight of the whole plasmid, although, in this gel system, resolution declines rapidly for fragments greater than 5×10^6 in molecular weight (Old, 1973; Sharp, Sugden and Sambrook, 1973). It is also seen that from the

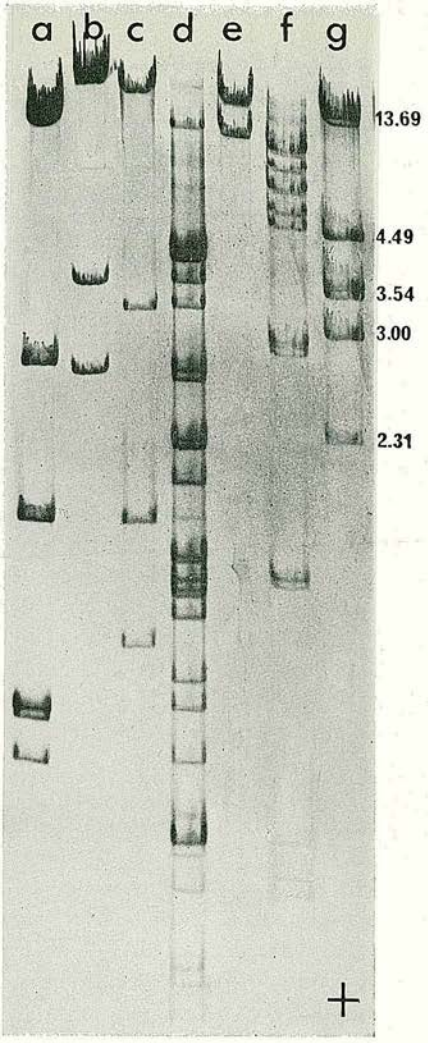

Fig. 1. Agarose gel electrophoresis of fragments produced by endonuclease digestion of plasmid DNA. The enzymes are endo *Ava*I (a, c, d) and endo R.*Eco*RI (b, e, f). The DNAs are: a, R245; b, R245; c, R6K; d, F; e, R6K; f, F. Track g shows fragments from λ DNA and their molecular weights multiplied by 10^{-6} (Allet *et al*, 1973). The scale is reduced about twofold

same substrate endo *Ava*I produces about twice as many fragments as does endo R.*Eco*RI. Having established that the fragments produced by endonucleolytic digestion of plasmid DNA can be resolved into distinctive patterns we then examined the patterns produced by closely related plasmids.

Comparison of R6, R100-1 and R1-19

The three R factors R6, R100 and R1 have been extensively characterised by molecular hybridisation and heteroduplex mapping, the two most powerful

Table 2. Approximate molecular weights of DNA fragments from R245, R6K and F

Plasmid	Enzyme	Number of fragments	Approximate molecular weights $\times 10^{-6}$	Sum of fragment molecular weights $\times 10^{-6}$
R245	endo *Ava*I	6[a]	15.2, 9, 2.0, 1.35, 1.30, 1.2	23.75
R245	endo R.*Eco*RI	3	23, 3.7, 2.8	29.5
R6K	endo *Ava*I	4	21, 3.4, 2.1, 1.5	28.0
R6K	endo R.*Eco*RI	2	17, 11.5	28.5
F	endo *Ava*I	29[b]	4.2, 4.1, 3.9, 3.6, 3.2, 2.85, 2.8, 2.7, 2.3, 2.2, 2.1, 1.85, 1.8, 1.75, 1.7, 1.65, 1.6, 1.4, 1.35, 1.15, 1.05, 1.0, 0.98, 0.95, 0.88, 0.78, 0.74, 0.70, 0.66	55.9
F	endo R. *Eco*RI	14[c]	10.0, 9.0, 7.8, 6.6, 5.2, 4.9, 2.95, 2.90, 2.85, 1.75, 1.70, 1.0, 0.93, 0.89	58.6

Molecular weight estimates were derived from a curve relating mobility to molecular weight constructed using DNA fragments of known molecular weight (Old, 1973).

[a] Three fragments with molecular weights less than 0.8×10^6 are not seen in Fig. 1.

[b] The uppermost band in Fig. 1, track d, is an incompletely digested fragment and is not included here.

[c] Two fragments with molecular weights less than 0.5×10^6 are not visible in Plate 1; see Fig. 2e.

analytical tools currently available (Guerry and Falkow, 1971; Sharp, Cohen and Davidson, 1973). Heteroduplex mapping shows that all of the DNA sequences of R100-1 and 85% of R1 sequences are present in R6 (Sharp, Cohen and Davidson, 1973). It is therefore useful to examine the fragment patterns produced after digestion of these plasmids. Fig. 2 shows that R100-1 and R6 DNA treated with endo *Ava*I (tracks a and b) produce very similar patterns with many coincident bands; however, they can still be distinguished by several band differences, which are arrowed in Fig. 2. In contrast, comparison of the fragment patterns produced by endo *Ava*I from R6 and R1-19 DNA (Fig. 2, tracks c and d) shows few common bands. This is somewhat surprising in view of the high degree of homology revealed by heteroduplex mapping between R1 and R6 and by hybridisation between R100 and R1 (73% homology; Guerry and Falkow, 1971). Possibly very minor changes in DNA sequence that destroy or create the recognition target sequence for the endonuclease are undetectable by these other techniques.

Comparison of F and FΔ (33—43)

In addition, we have examined the sex factor F and a mutant of it, FΔ (33—43), in which 10% of the DNA has been lost in a simple deletion event (Anthony *et al.*, 1974). Tracks e-h of Fig. 2 show a normal contrast photograph of the fluorescent DNA fragments derived from F and FΔ (33—43) by digestion with endo

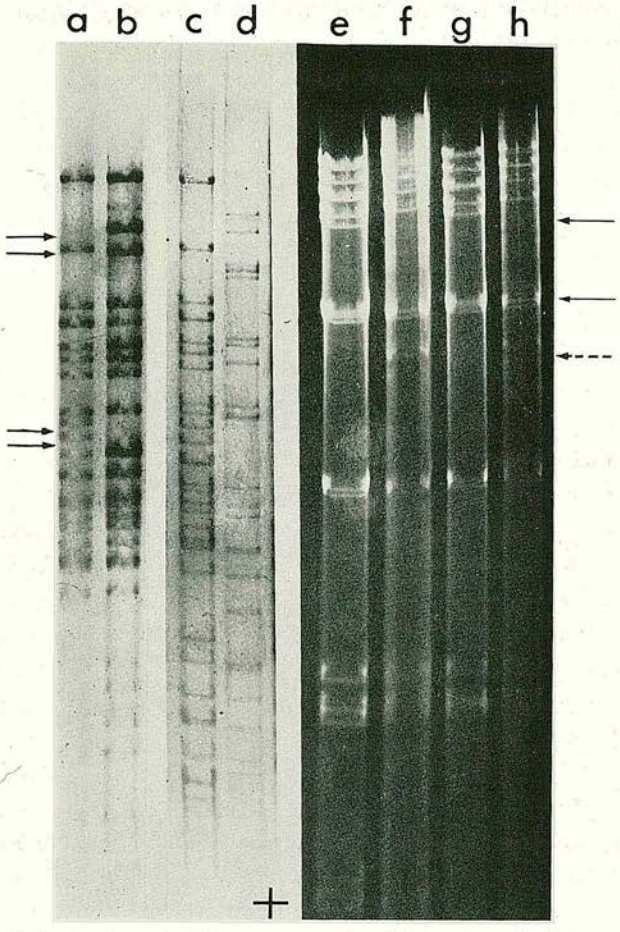

Fig. 2. Agarose gel electrophoresis of fragments produced by endonuclease digestion of plasmid DNA. Tracks a, b, c and d show fragments produced by endo *Ava*I from; a, R6; b, R100-1; c, R6; d, R1-19. Arrows indicate band differences between R100-1 and R6. The scale is reduced about twofold. Tracks e, f, g and h show fragments produced by endo R.*Eco*RI from F (e and g) and FΔ (33—43)(f and h). Tracks e and f contain twice as much DNA as g and h. Bands 1 and 2 (numbering from the top) run very close together. Bands 6 and 7 of the F digest are missing in the FΔ (33—43) digest and are marked by solid arrows. The broken arrow indicates the band in FΔ (33—43) not present in F. Reproduced at 0.9 × actual size

R.*Eco*RI. Two bands of molecular weights 4.9 and 2.95×10^6 (indicated by solid arrows in Fig. 2) present in the F digest are absent from the FΔ (33—43) digest, which contains a different band with a molecular weight of 1.6×10^6 (indicated by a broken arrow in Fig. 2). We estimate that the overall loss of DNA in the deletion mutant is 6.25×10^6 daltons. This is in agreement with the size of the deletion found by heteroduplex mapping, 6.6×10^6 daltons (Anthony *et al.*, 1974). We can thus conclude that fragments 6 and 7 (numbering from the top of the picture) of F DNA are adjacent and straddle the 33—43 kb region of F DNA in

the co-ordinate system of Sharp *et al.* (1972). The deletion event has occurred within fragments 6 and 7 and removed the endo R.*Eco*RI target site between them; a single new fragment is then generated when the DNA is digested with endo R.*Eco*RI.

Discussion

The number and size of fragments produced by cleavage of a circular plasmid DNA molecule with a specific endonuclease will depend upon the number and distribution of the target sequences broken by the enzyme. We have shown that plasmids give a characteristic band pattern when endonuclease-generated fragments are fractionated on agarose gels and that even very closely related plasmids can be readily distinguished by this technique. This approach provides a powerful test of the identity of two plasmid DNAs. The amount of DNA required is relatively small and the isolation procedure used here could be scaled down to isolate small (10 μg) quantities of unlabelled plasmid DNA. Alternatively, an even smaller-scale preparation to isolate radioactively labelled DNA could be used; the DNA bands could then be located by autoradiography of the gel. Also it may be possible to simplify the isolation procedure by using the DNA from cleared lysates (Clewell and Helinski, 1970) concentrated by ethanol precipitation. Such possibilities have not been systematically explored. Further modifications would allow isolation of individual bands and digestion with a second enzyme to yield extra information.

It is interesting to note that the parental F factor and its deletion mutant FΔ (33—43), which have been maintained in separate strains since at least 1953 (Lederberg and Lederberg, 1953; Bachmann, 1972) and which were shown to have diverged by 1963 (Schell *et al.*, 1963; Anthony *et al.*, 1974) have conserved the spatial arrangement of endo R.*Eco*RI target sites in over twenty years of independent subculture. Also, the distribution of the sites is such that there is a wide range of fragment sizes. This allows each fragment species to be resolved as a separate band. The analysis of partial digests may provide a means of mapping the fragments on the parental molecule.

The plasmids examined here all have molecular weights over 25×10^6. Restriction enzymes with shorter and hence more numerous target sequences exist (Smith and Nathans, 1973) and may be useful for smaller plasmids. It should also be noted that the band pattern will be as characteristic for plasmid aggregates (Clowes, 1972) as for the plasmid cointegrates studied here and that the method can be applied to the comparison of plasmids isolated from different bacterial species.

Finally, cleavage of DNA with endo R.*Eco*RI produces fragments which all have cohesive ends (Mertz and Davis, 1972). Cohen *et al.* (1973) and Chang and Cohen (1974) have shown that this allows manipulation of plasmid DNA *in vitro* and subsequent transformation back into *E. coli*. This offers a new approach to the study of the molecular biology of plasmids.

Acknowledgements. We thank K. Murray for the generous gift of restriction enzyme endo R.*Eco*RI. R. T. is an MRC scholar.

References

Allet, B., Jeppesen, P. G. N., Katagiri, K. J., Delius, H.: Mapping the DNA fragments produced by cleavage of λ DNA with endonuclease RI. Nature (Lond.) **241**, 120–122 (1973)

Anthony, W. M., Deonier, R. C., Lee, H. J., Hu, S., Ohtsubo, E., Davidson, N., Broda, P.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. IX. Note on the deletion mutant of *F*, *F*Δ (33—43). J. molec. Biol. (in press)

Bachmann, B. J.: Pedigrees of some mutant strains of *Escherichia coli* K-12. Bact. Rev. **36**, 525–557 (1972)

Bannister, D.: Restriction and modification controlled by resistance transfer factors. Ph.D. Thesis, Edinburgh University 1969

Chabbert, Y. A., Scavizzi, M. R., Witchitz, J. L., Gerbaud, G. R., Bouanchaud, D. H.: Incompatibility groups and the classification of fi⁻ resistance factors. J. Bact. **112**, 666–675 (1972)

Chang, A. C. Y., Cohen, S. N.: Genome construction between bacterial species *in vitro*: replication and expression of *Staphylococcus* plasmid genes in *Escherichia coli*. Proc. nat. Acad. Sci. (Wash.) **71**, 1030–1034 (1974)

Clewell, D. B., Helinski, D. R.: Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**, 4428–4440 (1970)

Clowes, R. C.: Molecular structure of bacterial plasmids. Bact. Rev. **36**, 361–405 (1972)

Clowes, R. C., Hayes, W.: Experiments in microbial genetics. Oxford: Blackwell 1968

Cohen, S. N., Chang, A. C. Y., Boyer, H. W., Helling, R. B.: Construction of biologically functional bacterial plasmids *in vitro*. Proc. nat. Acad. Sci. (Wash.) **70**, 3240–3244 (1973)

Curtiss III, R., Renshaw, J.: F⁺ strains of *Escherichia coli* K-12 defective in Hfr formation. Genetics **63**, 7–26 (1969)

Datta, N., Hedges, R. W.: Compatibility groups among fi⁻ R factors. Nature (Lond.) **234**, 222–223 (1971)

Egawa, R., Hirota, Y.: Inhibition of fertility by multiple drug-resistance factor in *Escherichia coli* K12. Jap. J. Genet. **37**, 66–69 (1962)

Grindley, N. D. F., Grindley, J. N., Anderson, E. S.: R factor compatibility groups. Molec. gen. Genet. **119**, 287–297 (1972)

Grindley, N. D. F., Humphreys, G. O., Anderson, E. S.: Molecular studies of R factor compatibility groups. J. Bact. **115**, 387–398 (1973)

Grinsted, J., Saunders, J. R., Ingram, L. C., Richmond, M. H.: Properties of an R factor which originated in *Pseudomonas aeruginosa* 1822. J. Bact. **110**, 529–537 (1972)

Guerry, P., Falkow, S.: Polynucleotide sequence relationships among some bacterial plasmids. J. Bact. **107**, 372–374 (1971)

Hayward, G. S., Smith, M. G.: The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. J. molec. Biol. **63**, 383–395 (1972)

Hedges, R. W., Datta, N.: R124, an fi⁺ R factor of a new compatibility class. J. gen. Micro. **71**, 403–405 (1972)

Hedges, R. W., Datta, N., Coetzee, J. N., Dennison, S.: R factors from *Proteus morganii*. J. gen. Micro. **77**, 249–259 (1973)

Kaiser, A. D., Hogness, D. S.: The transformation of *Escherichia coli* with deoxyribonucleic acid isolated from bacteriophage λ*dg*. J. molec. Biol. **2**, 392–415 (1960)

Kontomichalou, P., Mitani, M., Clowes, R. C.: Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. J. Bact. **104**, 34–44 (1970)

Lederberg, E., Lederberg, J.: Genetic studies of lysogenicity in *E. coli*. Genetics **38**, 51–64 (1953)

Mertz, J. E., Davis, R. W.: Cleavage of DNA by R1 restriction endonuclease generates cohesive ends. Proc. nat. Acad. Sci. (Wash.) **69**, 3370–3374 (1972)

Meynell, E., Datta, N.: Mutant drug resistance factors of high transmissibility. Nature (Lond.) **214**, 885–887 (1967)

Old, R. W.: Nucleotide sequences in defined regions of viral DNA. Ph.D. Thesis, Edinburgh University 1973

Radloff, R., Bauer, W., Vinograd, J.: A dye-bouyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. nat. Acad. Sci. (Wash.) **57**, 1514–1521 (1967)

Richmond, M. H.: Resistance factors and their ecological importance to bacteria and to man. Prog. in nucleic acid research and molec. biol. **13**, 191–248 (1973)

Schell, J., Glover, S. W., Stacey, K. A., Broda, P. M. A., Symonds, N.: The restriction of phage T3 by certain strains of *Escherichia coli*. Genet. Res. **4**, 483–484 (1963)

Sharp, P. A., Cohen, S. N., Davidson, N.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) and F factors. J. molec. Biol. **75**, 235–255 (1973)

Sharp, P. A., Hsu, M., Ohtsubo, E., Davidson, N.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. I. Structure of F-prime factors. J. molec. Biol. **71**, 471–497 (1972)

Sharp, P. A., Sugden, J., Sambrook, J.: Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochem. **12**, 3055–3063 (1973)

Smith, H. O., Nathans, D.: A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. J. molec. Biol. **81**, 419–423 (1973)

Watanabe, T., Ogata, C., Sato. S.: Episome mediated transfer of drug resistance in *Enterobacteriaceae*. VIII. Six-drug-resistance R factor. J. Bact. 88, 922–928 (1964)

Yoshimori, R. N.: A genetic and biochemical analysis of the restriction and modification of DNA by resistance transfer factors. Ph.D. Thesis. University of California (1971)

Communicated by Ch. Auerbach

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Molec. gen. Genet. 160, 347–351 (1978)

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Molecular Relationships of Degradative Plasmids Determined by in situ Hybridisation of Their Endonuclease-Generated Fragments

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Summary. Plasmid inter-relationships were studied by hybridisation of a radioactively labelled DNA probe to endonuclease-derived fragmentation patterns of plasmids bound to a nitrocellulose filter. The degradative plasmids SAL and NAH were found to be very closely related, but probably one did not give rise to the other by just a single deletion or insertion. Relationships between SAL and other degradative plasmids are complex; substantial homology was found with TOL and other plasmids encoding toluate dissimilation and significant homology was found with OCT.

Introduction

Molecular relationships between different plasmid species have been investigated by electron-microscopy of heteroduplex molecules (Sharp et al., 1973) and by comparison of fragmentation patterns after digestion with site-specific endonucleases (Thompson et al., 1974; Duggleby et al., 1977). Heteroduplexes can provide a wealth of information on sequence relationships but analysis of the data can be difficult unless reference points, provided by base-sequence insertion or deletion, are used as internal markers. Only closely related plasmids yield similar patterns of fragment sizes after endonuclease digestion; even when a limited relatedness appears to exist, ambiguity may be introduced when totally different fragments have indistinguishable electrophoretic mobilities.

We have developed a method whereby information is provided both by the fragmentation pattern and by molecular hybridisation. This involves in situ hybridisation of single-stranded radioactively-labelled plasmid DNA against the endonuclease-generated fragments of plasmid DNA that have been denatured and transferred to a nitrocellulose filter by the method of Southern (1975). We have tested the efficacy of this method by investigating the relationships of a number of degradative plasmids isolated from soil pseudomonads.

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Materials and Methods

Bacterial Strains

The toluate-utilising strains used here as sources of plasmid DNA have been described previously (Duggleby et al., 1977). Other strains and the plasmids they harbour are given in Table 1.

Table 1. Bacterial strains

Strain	Description	Plasmid	Reference
PpG7	*P. putida* biotype A (ATCC17485)	NAH	Dunn and Gunsalus, 1973 Barnsley, 1976
ATCC17483	Unclassified fluorescent pseudomonad	Not previously reported.	Barnsley, 1976
AC165	*P. aeruginosa* PAC; prototrophic transconjugant	SAL	Chakrabarty, 1972
PpS5	*P. putida* PpGl; *met*$^-$, *alcA*$^-$	OCT	Grund et al., 1975

Plasmid Isolation, Digestion and Electrophoresis

The materials and procedures used in isolation, endonuclease digestion and electrophoresis of plasmid DNA have already been described (Duggleby et al., 1977).

0026-8925/78/0160/0347/$01.00

Transfer of Plasmid DNA Fragments to Nitrocellulose Filter

DNA fragments were transferred from 1% agarose gels (3 mm thick and up to 20 cm in planar dimensions) to nitrocellulose filters by the method of Southern (1975) slightly modified. Denaturation (20–30 min) and neutralization (40 min) were carried out in flat trays, with occasional mixing to ensure that the gel was at all times submerged in solution. Between denaturation and neutralization the gel was rinsed with neutralization solution. Transfer of DNA from the whole gel was carried out in an apparatus constructed from a horticultural polystyrene seed tray (36 × 22 cm) upon which a sheet of glass (40 × 20 cm) was laid. Five sheets of Whatman 3 MM paper were draped over the glass to form a wick in contact with the 20 × SSC buffer (Southern, 1975) held in the tray. The denatured/neutralized gel was laid upon this buffer-moistened pad; under the peripheral few millimetres of the gel, 2 cm-wide strips of heavy gauge polythene sheeting were inserted between the paper and the gel. In this way a continuous perimeter was formed around the gel, thus preventing liquid short-circuits due to either capillary action or from the sagging of the overlaid Whatman paper (to be applied subsequent to this). Perspex support strips (3 mm thick) were laid alongside the gel, but separated by several millimeters from it. A sheet of nitrocellulose filter (type BA 85, Schleicher & Schüll, Dassel, W. Germany), precut to the exact length but one centimetre wider than the gel, was moistened with 2 × SSC buffer and then laid carefully upon the gel so that it overlapped and rested on the longitudinal support strips. Care was taken to avoid trapping air bubbles between the gel and the paper and between the nitrocellulose filter and the gel. Several sheets of moistened (20 × SSC) Whatman paper were likewise laid upon the nitrocellulose filter; in turn, sheets of dry Whatman paper were laid upon these to form a pile 20 cm high. This was weighed down with a glass sheet and the whole was covered with cling film. After six hours the pile was removed, leaving only one moistened paper sheet covering the nitrocellulose filter. The perspex support-strips were then replaced by thinner ones (1.5 mm thick) to compensate for the shrinking gel, and a new pile of dry Whatman paper was placed on top. Further transfer (16 h) ensued. By this time the gel had shrunk so much that for the subsequent (and final) 12 h transfer it was necessary to replace each perspex support strip by four strips of heavy-gauge polythene sheeting. The paper absorbant was replaced as the pile became wet. After transfer the gel was removed from the nitrocellulose filter by soaking in 2 × SSC buffer for ten minutes. The filter was then dried at 37° C for half an hour before baking (80° C) in vacuo for 2 h. Confirmation that DNA had transferred from the gel was provided by restaining it in ethidium bromide solution (0.5 μg/ml) and photographing it as previously. Those DNA fragments larger than about 5 Md transferred poorly and substantial proportions of these species remained behind in the gel.

Radioactive Labelling of Plasma DNA by "Nick Translocation"

The plasmid DNA probe was radioactively labelled to a high specific activity by "nick translocation" (alternatively termed "nick-translation"; Maniatis et al., 1975) after initial random nicking of the covalently-closed plasmid DNA by pancreatic DNase. The "nick translation" reaction mixture (Maniatis et al., (1975), but without bovine serum albumin) was made up with approximately 1 μg plasmid DNA in sterile 1.5 ml polypropylene tubes (No. 39/10, W. Sarstedt (U.K.,) Leicester, LE4 7 LZ) using 10 μCi{α $-^{32}$P}dGTP (10 μl of 8 μM ethanolic solution, supplied by Radiochemical Centre, Amersham, dried for 2 h in vacuo over conc. H_2SO_4) and unlabelled deoxyribonucleoside triphosphates of the other three bases (Boehringer-Mannheim, W. Germany). Before the addition of one unit of *E. coli* DNA polymerase I (highest purity, Boehringer-Mannheim, W. Germany), 2 μl of 1 μg/ml pancreatic DNase I (freshly diluted in water from a frozen stock solution, also in water, of 1 mg/ml; Sigma crude bovine pancreatic DNase I) was added and nicking of DNA was allowed to proceed for two minutes at room temperature. During the subsequent incubation with DNA polymerase at 15°C, samples were taken at hourly intervals to measure uptake of ^{32}P into acid-precipitable material. After incubation for four hours the reaction was terminated by the addition of 0.5 ml of pre-equilibrated (TNE buffer: 10 mM Tris; 10 mM NaCl; 2 mM EDTA; pH 8.0) phenol (distilled under nitrogen and preserved from peroxidation by storing with approximately 0.01% 8-hydroxyquinoline at 4°C in the dark). Carrier nucleic acid was added as 10 μl yeast RNA (10 mg/ml; Sigma, type III). The reaction mixture was extracted twice with this phenol, twice with chloroform-propan-2-ol (24:1) and twice with diethyl ether; the remaining ether was then removed by gentle aspiration at 37° C. The nucleic acid was separated from the reaction substrates by chromatography on a 7 ml column of Sephadex G-50 in TNE buffer. Two-drop fractions were collected and radioactivity was detected with a hand-held Geiger-Müller monitor. The position of the reaction substrates was indicated by the co-chromatography of a small amount (sufficient for colouration) of Orange G dye in the sample. Fractions were counted by Cherenkov radiation and the peak activity, comprising approximately 40% of the total labelled nucleic acid, was pooled and stored at −20°C. Total ^{32}P counts were determined by liquid scintillation (butyl-PBD, 0.5% in toluene) as acid-precipitable (5% trichloracetic acid) material. Samples of this labelled nucleic acid, containing 10^6 cpm, were denatured for five minutes in a boiling water bath and cooled rapidly in iced water before use as single-stranded probes for hybridisation with the DNA bound to the nitrocellulose filter.

Hybridisation of Plasmid Probe to Nitrocellulose-bound Single-stranded DNA

Nitrocellulose filters were prepared for hybridisation by incubation in 100 ml measuring cylinders according to the method of Denhardt (1966). The hybridisation solution contained 10^6 cpm of single-stranded DNA probe, the ingredients of the pre-incubation solution in 4 × SSCP (SSCP is 120 mM NaCl, 15 mM sodium citrate, 15 mM KH_2PO_4, 1 mM EDTA; pH 7.2), and 0.2% (w/v) sodium dodecyl sulphate (to reduce non-specific binding of DNA to the nitrocellulose filter). Hybridisation was carried out in siliconized (Repelcote; Hopkin and Williams) 250 ml measuring cylinders with the nitrocellulose filter wrapped around a nylon plug insert. In this way the volume of the solution was kept to 10 ml or less. It was found that a greater degree of hybridization occurred if the side of the filter to which the DNA was bound (i.e. that which had been in contact with the agarose gel) faced inwards, since the filter had a tendency to cling to the glass. A few drops of paraffin oil were layered over the hybridization mixture to reduce surface evaporation and the whole was incubated in a covered water bath at 65°C for 40 h. After half an hour, and at 12-hourly intervals throughout the incubation, the nylon plug was rotated to dislodge air bubbles from the filter. The filter was then washed in 0.05% SDS in 4 × SSC buffer at 65°C for 2 h and then in 4 × SSC buffer alone at 65°C for 30 min. It was then briefly rinsed in 2 × SSC buffer at room temperature and dried at 37°C for an hour prior to exposure to pre-exposed photographic film (XHl, X-omat, Kodak-Eastman) next to an intensification screen (Ilford Fast Tungstate) at −70° C (Laskey and Mills, 1977).

Results

Homology of SAL and NAH Plasmids

The degradative plasmids NAH and SAL, which encode the degradation of naphthalene and salicylate respectively, sediment at similar velocities through alkaline sucrose gradients (Johnston and Gunsalus, 1977). Both plasmids encode the dissimilation of salicylate, an intermediate of naphthalene catabolism, *via* the *meta*-cleavage of catechol (Chakrabarty, 1972; Dunn and Gunsalus 1973; Barnsley, 1976). We find that they also yield very similar *Eco*RI endonuclease fragmentation patterns (Fig. 1a, tracks 7 and 8). From the numbers and sizes of fragments we calculate that the sizes of NAH and SAL are 46 Md and 42 Md respectively. Each has five fragments that do not correspond in size to any from the other plasmid. In both, Figure 1a tracks 7 (the NAH digest) and 8 (the SAL digest), these comprise the two largest fragments and the three bands marked with arrows. In track 8 the middle arrow indicates the position of a doublet (determined by densitometry) where the corresponding SAL band represents only a single size species.

Initial investigations showed that the SAL plasmid DNA "probe" hybridised to all fragments of SAL bound to a nitrocellulose filter and to all, except the three labelled with arrows in Figure 1a, of the NAH plasmid. These three are among the five for which there are no size-analogues in the SAL fragmentation pattern. In the reciprocal experiment, probe of the NAH plasmid hybridised with all fragments of the SAL plasmid. In other experiments we have shown that both on fragmentation pattern and by the criterion of hybridisation, plasmid DNA from a second Nah^+ strain (ATCC17483) is indistinguishable from that of strain PPG7. This explains why the biochemistry of naphthalene dissimilation by these two strains (Barnsley, 1976) is so similar.

Homology of SAL with Other Degradative Plasmids

Figure 1 also shows the extent of hybridisation between SAL probe DNA and digests of other de-

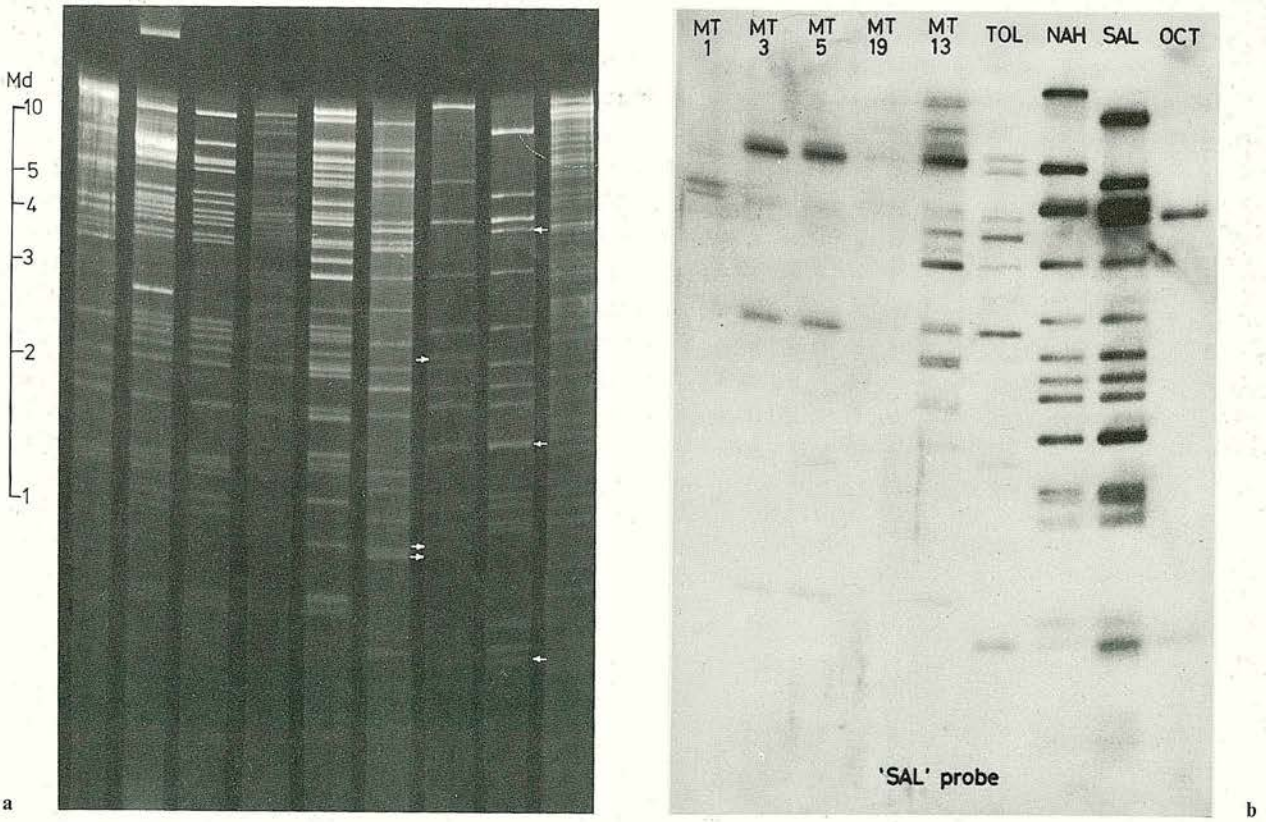

Fig. 1a and b. Fragmentation patterns of endonuclease *Eco*RI-digested degradative plasmids (as labelled in **b**) after agarose gel electrophoresis and staining with ethidium bromide **a**. The scale indicates the size of the DNA fragments in Megadaltons (Md: molecular weight $\times 10^{-6}$) previously determined with reference to the EcoR1-generated fragments of phage λ DNA (Duggleby et al., 1977). **b** shows the autoradiograph derived from this electrophoretogram after denaturation, transfer to a nitrocellulose filter and hybridisation with ^{32}P-labelled DNA of the degradative plasmid SAL. The arrowed bands are referred to in the text

Table 2. Number of bands from *Eco*RI-digested plasmid DNAs that show some homology with SAL. Note that some bands contain more than one fragment-species

Strain	AC165	PpG7	PpS5	PaW1	MT1	MT3	MT5	MT13	MT19
Plasmid	SAL	NAH	OCT	TOL (pWW0)	pWW1 pWW2	pWW3 pWW4	pWW5	pWW13	pWW19
Number of bands apparent by fluorescence	19	20	42	23	14	28	27	37	33
Bands with some homology	19	17	2	11	6	11	10	18	11

gradative plasmids. Two different periods of autoradiographic exposure (18 h and 6 days) were used. The longer period was necessary to reveal lesser amounts of hybridisation due to a smaller input of DNA (MT 19), a diminished degree of homology or inefficient transfer of DNA from the gel to the filter. The SAL *vs* SAL hybridisation provides an internal control on the degree of hybridisation for each fragment. This allows corrections to be made for the poor transfer of large fragments and inefficient hybridisation of small fragments (Southern, 1975). The fact that there is relatively little non-specific binding was apparent in the hybridisation of SAL with OCT, where hybridisation occurred in two bands only, even after six days autoradiographic exposure.

A further observation was that a common fragment of the plasmids from MT3, MT5 and MT13 (Fig. 1b) and MT1 and MT19 (six day exposure) showed strong homology with SAL. Lesser degrees of homology were evident in other fragments. The numbers of fragments in which there appears to be homology with SAL are given in Table 2.

In other experiments we have used plasmids pWW19 and TOL as probes. Here we found that pWW19 had homology not only with the plasmids from strains MT1, MT3, MT5 and MT19 (as suggested by Duggleby et al., 1977) but also with pWW13 (from MT13), TOL, SAL and NAH. The larger OCT fragment that showed homology with SAL also hybridised with this probe. TOL has homology with the plasmids of the MT1 set of strains (see above) and also with pWW13, SAL and NAH, but not with OCT. Details of these analyses will be published separately.

Discussion

The method presented here provides more information on homology between plasmid molecules than does simple hybridisation, in that regions of homology are specified. Where several but not all fragments from a given plasmid show homology with another plasmid, the most economical hypothesis is that they were adjacent in the whole plasmid. Conversely, when the sequence of fragments becomes known, it will be possible to infer the molecular relationships of regions spanning several fragments.

Often parts only of fragments will be homologous with the probe. Therefore it is not possible to determine the overall homology between any two plasmids merely by adding up the sizes of the fragments that show some homology. However, if an internal control (hybridisation against fragments of the same plasmid) is included to show the relative efficiency of transfer of and hybridisation to DNA fragments of different sizes, it may be possible to determine the approximate extent of homology. For this purpose it is also necessary to have at least one band which is known or assumed to have complete homology with DNA of the probe.

The finding that the NAH plasmid is about 4 Md larger than the SAL plasmid might suggest that SAL has descended from NAH by simple loss of the genes specifying the steps of naphthalene dissimilation as far as salicylate. However, although the two plasmids are clearly closely related, their evolutionary relationship must be more complex, since each yields five fragments not present in the digest of the other. If SAL arose from NAH by a simple deletion (or NAH from SAL by a simple insertion) one would expect SAL to have only a single fragment not present in NAH. Nevertheless, there is no evidence that SAL contains sequences not present in NAH, although we have demonstrated that the converse is true in that three fragments of the NAH digest do not hybridise to the SAL probe.

The two putative NAH plasmid species studied here derive from two naphthalene-dissimilating fluorescent pseudomonads classified into different biotypes (Stanier et al., 1966) that were both isolated at Rothampstead, England. The plasmids are indistinguishable by our methods. It also emerges that some sequences of the other degradative plasmids

have homology with parts of the SAL plasmid (the origin of which has not been reported). Indeed, all the plasmids tested here, even OCT, show some homology with SAL. These findings, and the relatedness of SAL and the NAH plasmids, support the suggestion (Sharp et al., 1973; Heffron et al., 1975; Santos et al., 1975; Duggleby et al., 1977) that some sequences, including transposons, are widespread in nature.

Our preliminary investigation of the plasmids encoding toluate-dissimilation, using TOL and pWW19 probes, shows more complex inter-relationships. It might be possible to use cloned fragments, or DNA of deleted plasmids that have lost functions, to bring together knowledge on structural and functional relationships.

Acknowledgments. A.L.H. was a British Council Fellow. We thank E. Barnsley for strains ATCC 17483 and PpG7, A. Chakrabarty for strain AC165 and J. Shapiro for strain PpS5. We are very grateful to K. Peden for demonstrating and helping us with the methods described in this paper.

References

Barnsley, E.A.: Role and regulation of the *ortho* and *meta* pathways of catechol metabolism in pseudomonads metabolizing naphthalene and salicylate. J. Bact. **125**, 404–408 (1976)

Chakrabarty, A.M.: Genetic basis of the biodegradation of salicylate in *Pseudomonas*. J. Bact. **112**, 815–823 (1972)

Denhardt, D.T.: A membrane-filter technique for the detection of complementary DNA. Biochem. biophys. Res. Commun. **23**, 641–646 (1966)

Duggleby, C.J., Bayley, S.A., Worsey, M.J., Williams, P.A., Broda, P.: Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bact. **130**, 1274–1280 (1977)

Dunn, N.W., Gunsalus, I.C.: Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bact. **114**, 974–979 (1973)

Grund, A., Shapiro, J., Fennewald, N., Bacha, P., Leahy, J., Markbreiter, K., Nieder, M., Toepfer, M.: Regulation of alkane oxidation in *Pseudomonas putida*. J. Bact. **123**, 546–556 (1975)

Heffron, F., Sublett, R., Hedges, R.W., Jacob, A., Falkow, S.: Origin of the TEM Beta-lactamase gene found on plasmids. J. Bact. **122**, 250–256 (1975)

Johnston, J.B., Gunsalus, I.C.: Isolation of metabolic plasmid DNA from *Pseudomonas putida*. Biochem. biophys. Res. Commun. **75**, 13–19 (1977)

Laskey, R.A., Mills, A.D.: Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Letters **82**, 314–316 (1977)

Maniatis, T., Jeffrey, A., Kleid, D.G.: Nucleotide sequence of the rightward operator of phage λ. Proc. nat. Acad. Sci. (Wash.) **72**, 1184–1188 (1975)

Santos, D.S., Palchaudhuri, S., Maas, W.K.: Genetic and physical characteristics of an enterotoxin plasmid. J. Bact. **124**, 1240–1247 (1975)

Sharp, P.A., Cohen, S.N., Davison, N.: Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II Structure of drug resistance (R) factors and F factors. J. molec. Biol. **75**, 235–255 (1973)

Southern, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. molec. Biol. **98**, 503–517 (1975)

Stanier, R.Y., Palleroni, N.J., Doudoroff, M.: The aerobic pseudomonads: a taxonomic study. J. gen. Microb. **43**, 159–271 (1966)

Thompson, R., Hughes, S.G., Broda, P.: Plasmid identification using specific endonucleases. Molec. gen. Genet. **133**, 141–149 (1974)

Communicated by Ch. Auerbach

Received January 6, 1978

JOURNAL OF BACTERIOLOGY, June 1979, p. 1036–1037
0021-9193/79/06-1036/02$02.00/0

Vol. 138, No. 3

R Plasmids R91 and R91a from *Pseudomonas aeruginosa* Share Only the Gene for Carbenicillin Resistance

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Received for publication 7 March 1979

Plasmid R91a of *Pseudomonas aeruginosa* strain 9169 is homologous with RP1. Plasmid R91 carried by the same strain is related in the Tn*1* region but is otherwise unrelated to R91a.

Pseudomonas aeruginosa strain 9169, isolated at the Birmingham Accident Hospital in 1969 by Lowbury et al. (10), was reported to contain a plasmid expressing resistances to carbenicillin, kanamycin, and tetracycline in *Escherichia coli*, but resistance only to carbenicillin in *Pseudomonas* strains (2). Further physical and genetic studies have now revealed that strain 9169 contains two plasmids which differ with respect to their molecular sizes and restriction endonuclease recognition sites (9). One is an incompatibility group (Inc) P-1 plasmid, R91a, which determines resistance to carbenicillin, kanamycin, and tetracycline. The size of plasmid R91a, 39 megadaltons, as determined by electron microscopy, is similar to that of the prototype Inc P-1 plasmid RP1; like RP1, it has only a single *Eco*RI recognition site. However, plasmid R91a is unusual among Inc P-1 plasmids in that its transfer functions are expressed with barely detectable frequency. The other plasmid present in strain 9169, R91, confers resistance to carbenicillin only and has been included in a new *Pseudomonas* Inc group, P-10. It has a smaller molecular size than R91a (35 megadaltons compared with 39 megadaltons) and has at least nine *Eco*RI recognition sites. A more highly transmissible derivative of R91, R91-5, allows multiplication of phages PRD1, PR3, and PR4. Although these phages also lyse RP1-carrying strains, they also bind to pili specified by plasmids of other Inc groups (1). In contrast, phages PRR1 and Pf3, which attach specifically to P-1 pili, do not lyse a strain carrying R91 (9).

We were interested in studying the interrelationships among RP1, R91, and R91a. This was done by hybridization of radioactively labeled probes of each of these DNA species to endonuclease-cleaved fragmentation patterns of the other plasmids bound to nitrocellulose filters by Southern transfer (7, 11). Plasmid R91a, in an *E. coli* host strain, J53, and plasmid R91, in *P. aeruginosa* strain PU21 (9), were provided by G. A. Jacoby. Plasmid DNA was prepared from 1-liter cultures grown to log phase in L broth by the technique of Guerry et al. (5). After removal of the bulk of the chromosomal DNA by salt-sodium dodecyl sulfate precipitation, plasmid DNA was concentrated by polyethylene glycol precipitation (8). Final purification was by ethidium bromide-cesium chloride equilibrium gradient centrifugation at 38,000 rpm for 40 h in a type Ti 50 rotor on a Beckman model L2-50 ultracentrifuge. Plasmid RP1 DNA was kindly supplied by J. M. Watson.

Plasmid DNA was digested by restriction endonucleases *Pst*I and *Eco*RI (MRE, Porton Down, Wilts.). Digestion with *Pst*I was carried out for 1 h at 37°C in 6 mM Tris-hydrochloride (pH 7.4)–6 mM NaCl–0.5 mM dithiothreitol, after which the NaCl concentration was increased to 100 mM and *Eco*RI restriction was performed for another hour. Electrophoresis of plasmid DNA in 1% agarose gels was essentially as described by Duggleby et al. (3), except horizontal gels were used. Transfer of DNA fragments to nitrocellulose filters (11), radioactive labeling of plasmid DNA by "nick translocation," and hybridization of the plasmid probe to nitrocellulose-bound single-stranded DNA have been described previously (7, 11).

Double digests of both RP1 and R91a with restriction endonucleases *Pst*I and *Eco*RI gave rise to seven fragments which could be resolved by electrophoresis on 1% agarose gels. The restriction patterns obtained for the two plasmid DNA species were identical. On the other hand, the R91 DNA gave a different fragmentation pattern. At least 15 bands were resolved, some of which, as deduced from densitometric traces of gel photographic negatives, consisted of two fragment species having the same mobilities. Only two bands in the R91 digest had mobilities identical to equivalent bands in the RP1 and R91a fragmentation patterns. The total of all of the fragments in the R91 digest gave a value of about 33 megadaltons as the size of the whole plasmid. This value compares with the size of 35

megadaltons (9) cited above.

When radioactively labeled RP1 DNA was hybridized with the endonuclease-derived fragments of RP1, R91, and R91a DNAs, it was observed (Fig. 1) that it hybridized strongly to each of the RP1 and R91a fragments.

When R91a DNA was used as the probe, it also hybridized strongly to all of the RP1 and R91a fragments. Therefore, RP1 and R91a are also indistinguishable at this level of analysis. In contrast, only four bands of the R91 pattern hybridized to the RP1 probe. The two bands which hybridized most strongly corresponded in mobility to RP1 bands E and G. Also, only four bands in the RP1 and R91a patterns hybridized to the R91 probe, and again only two bands showed strong homology. These corresponded to bands E and G in the RP1 pattern; relatively less hybridization occurred with bands A and D, indicating less homology.

Reference to the restriction map of plasmid RP1 (4) shows that fragments E and G, together with small portions of fragments A (adjacent to G) and D (adjacent to E), correspond to the transposable element Tn*1*. Therefore, our results lead us to conclude that the homology between R91 and RP1 that is detectable by our methods is limited to this transposon. Both R91 and R91a are known to determine a TEM-2-type beta-lactamase (9), an enzyme known to be carried by Tn*1* (6). Our results therefore confirm the conclusion of Jacoby et al. (9) that R91 originated by transposition of Tn*1* to another plasmid in the Birmingham *P. aeruginosa* strains, where intensive use of carbenicillin led to the emergence of the carbenicillin resistance strain 9169.

We thank G. Jacoby for strains and discussions and the Medical Research Council for support.

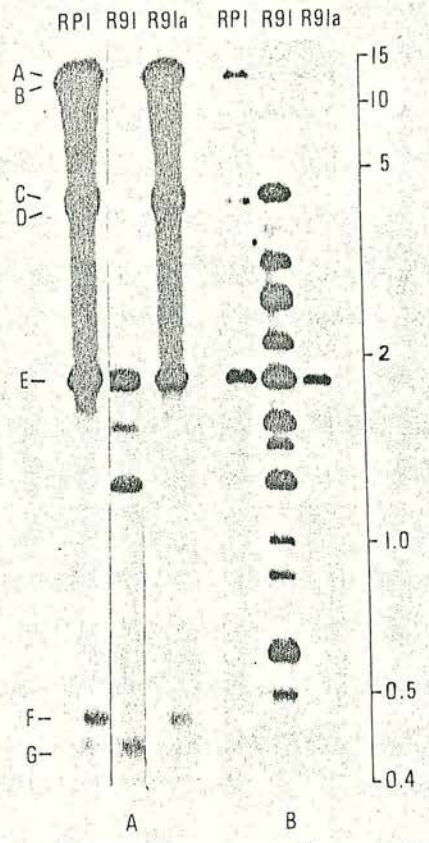

FIG. 1. *Extent of hybridization between RP1 and R91 DNA probes against the digests of RP1, R91, and R91a DNAs. The fragments released by digestion of RP1, R91, and R91a DNAs with a mixture of endonucleases EcoRI and PstI were resolved by electrophoresis in 1% agarose gels, denatured, transferred to nitrocellulose filters, and hybridized with (A) ^{32}P-labeled RP1 DNA and (B) ^{32}P-labeled R91 DNA. (A) is overexposed so as to reveal homology of this probe with the smaller fragments. The scale indicates the sizes of the DNA fragments in megadaltons. The letters identify the RP1 fragments in descending order of size.*

LITERATURE CITED

1. **Bradley, D. E.** 1977. Pili and associated bacteriophages of *Pseudomonas aeruginosa*, p. 127–133. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
2. **Chandler, P. M., and V. Krishnapillai.** 1974. Phenotypic properties of R factors of *Pseudomonas aeruginosa*: R factors transferable only in *Pseudomonas aeruginosa*. Genet. Res. 23:596–603.
3. **Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda.** 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. 130:1274–1280.
4. **Grinsted, J., P. M. Bennett, and M. H. Richmond.** 1977. A restriction enzyme map of R-plasmid RP1. Plasmid 1:34–37.
5. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064–1066.
6. **Hedges, R. W., and A. E. Jacob.** 1974. Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. 132:34–40.
7. **Heinaru, A. L., C. J. Duggleby, and P. Broda.** 1978. Molecular relationships of degradative plasmids determined by *in situ* hybridisation of their endonuclease-generated fragments. Mol. Gen. Genet. 160:347–351.
8. **Humphreys, G. O., G. A. Willshaw, and E. S. Anderson.** 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochim. Biophys. Acta 383:457–463.
9. **Jacoby, G. A., R. Weiss, T. R. Korfhagen, V. Krishnapillai, A. E. Jacob, and R. W. Hedges.** 1978. An explanation for the apparent host specificity of *Pseudomonas* plasmid R91 expression. J. Bacteriol. 136: 1159–1164.
10. **Lowbury, E. J. L., A. Kidson, H. A. Lilly, G. A. J. Ayliffe, and R. J. Jones.** 1969. Sensitivity of *Pseudomonas aeruginosa* to antibiotics: emergence of strains highly resistant to carbenicillin. Lancet ii:448–452.
11. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

(Reprinted from Nature, Vol. 280, No. 5720, pp. 338-339, July 26 1979)

The relationship of degradative and resistance plasmids of *Pseudomonas* belonging to the same incompatibility group

THE fluorescent pseudomonads include *Pseudomonas* which can cause serious infections in man, and soil bacteria such as *P. putida*. Resistance (R) plasmids have been found in strains of both species and degradative (D) plasmids have been demonstrated in strains of the latter species. Where such plasmids can be transferred by conjugation to a common host strain, it has been possible to establish whether they can stably co-exist. Inability to co-exist (incompatibility) is the basis of their classification[1,2]. It has recently been suggested that both the IncP-2 and IncP-9 groups contain both R and D plasmids[2,3]. In addition the IncP-2-plasmids are both very large[4]. Among R plasmids of enterobacteria, incompatibility is generally an indication of evolutionary relationship in that the DNAs of incompatible plasmids show greater homology with each other than with those of compatible plasmids[5]. However, cases of incompatible plasmids lacking significant homology have also been reported[5,6]. We have examined the extent to which two R plasmids from *P. aeruginosa* and two D plasmids, one from *P. putida* and the other from *P. aeruginosa* (all assigned to the IncP-9 group[3,15,16]) share common sequences. The results suggest that plasmids of the same incompatibility group are not necessarily closely related.

The plasmids used in this study are described in Table 1. Plasmid pMG18 carries the same resistance determinants as R2, plus several additional ones. Plasmids NAH and TOL encode the degradation of naphthalene and toluene respectively. In the course of both degradations, catechol is cleaved and degraded via the *meta* (α-ketoacid) pathway. However, the R plasmids have no known function in common with the D plasmids.

Overall homology was established by DNA : DNA hybridisation on nitrocellulose filters[17]. Plasmid pMG18 contained sequences that were homologous with most, if not all, of R2 (Table 2). The extent of homology in the reciprocal hybridisation (67%) was close to that expected (69%) from this experiment, given that pMG18 is larger than R2 (100 kilobases compared with 73 kilobases).

TOL DNA showed significant homology with both R plasmids (Table 2). Given that the sizes of TOL and R2 are 117 and 73 kilobases respectively, the TOL : R2 hybridisation showed that the two plasmids shared sequences of DNA equivalent to about 11 kilobases (the average of 117×0.11 and 73×0.14).

Table 1 Plasmids used in this study

Plasmid	Original host strain	Host strain in this work	Phenotype	Approximate size of plasmid DNA (kilobase-pairs)†	Ref.
R2	*P. aeruginosa*	PU21	CbSmSu	73	7
pMG18	*P. putida*	PU21	CbGmKmSmSuHg	100	8
NAH	*P. putida*	PpG7	Nah	72	9, 10
TOL	*P. putida*	PaW1	Tol	117	11

Abbreviations: Cb, Gm, Km, Sm, Su, Hg: resistance respectively to carbencillin, gentamicin, kanamycin, streptomycin, sulphonamide and Hg^{2+}. Nah, Tol: ability to use naphthalene and ability to utilise toluene and *m*- and *p*-xylenes.

* R2 and pMG18 were transferred from *P. aeruginosa* strain PU21[12], which carries cryptic plasmids[13], to a plasmid-free *P. putida* strain, AC34[14].

† The plasmid sizes were estimated from the sum of the sizes of the fragments derived from *Eco*RI cleavage and resolved on agarose gels. Fragments of λ DNA run on the same gels were used as reference markers.

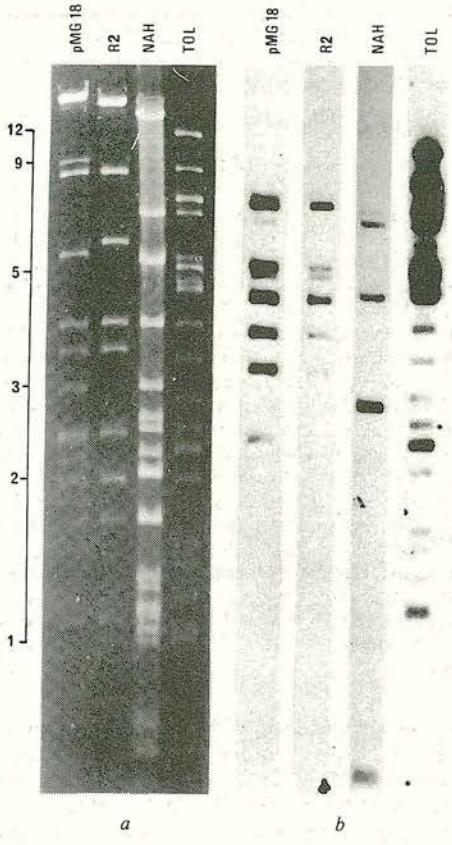

Fig. 1 *a* Banding patterns of *Eco*RI-digested plasmid DNA after electrophoresis on 1% agarose gels and staining with ethidium bromide. *b* Autoradiographs obtained by transferring *Eco*RI-digested fragments of TOL DNA to nitrocellulose filters by the Southern blot technique, and hybridising with the four different ^{32}P-labelled plasmid probes[10]. Probe DNA was labelled by 'nick translation' with [α^{32}P]-dCTP and sheared by sonication to an average length of 500 base-pairs. Hybridisation was performed in Denhardt's solution[20] at 65 °C for 18 h. Autoradiography was carried out for 24 h and then for a further 3 days to reveal weakly hybridising bands. The autoradiographs presented here were exposed for 24 h.

Similarly, we calculated that the DNA sequences common to TOL and pMG18 are equivalent to 20 kilobases (117×0.17 and 100×0.20). However, the R plasmids shared much less homology with NAH; here, the common sequences were only equivalent to about 3 kilobases.

Previous data had suggested that NAH and TOL were related[10]. We found that the extent of this relationship was small. The degree of reassociation of TOL DNA probe with unlabelled NAH DNA was $12 \pm 2\%$, and the reciprocal hybridisation gave $8 \pm 3\%$ reassociation. Thus, we calculated the sequences common to both plasmids as being equivalent to only 9 kilobases (72×0.12 and 117×0.08).

To localise the related segments of the R and D plasmids and to confirm that the observed homology between NAH and the R plasmids was significant, a series of hybridisations was performed, using radioactively-labelled probe DNAs of the plasmids and Southern blots of endonuclease-generated fragments of unlabelled plasmid DNAs[10].

The close similarity in size between most of the R2 fragments and a series of pMG18 fragments again suggested that R2 was very closely related to pMG18 (Fig. 1*a*). This was borne out by autoradiography, which showed that all R2 bands hybridised with pMG18 probe, and all but three bands in the pMG18 pattern showed homology with R2 probe (data not shown).

In the filter hybridisations described above, pMG18 showed more homology with TOL than did R2. As expected from this

Table 2 Homology among R and D plasmids of *Pseudomonas* belonging to the IncP-9 incompatibility group

Total unlabelled DNA from strain-carrying plasmid	Percentage of reassociation with labelled plasmid DNA from:			
	TOL	NAH	R2	pMG18
TOL	100±6	12±2	14±3	20±2
NAH	8±3	100±2	5±1	2±1
R2	11±3	4±1	100±3	67±2
pMG18	17±4	6±2	98±3	100±2

Plasmid DNA was isolated by SDS lysis followed by NaCl–SDS precipitation of chromosomal DNA[18] and purified by CsCl-ethidium bromide density ultracentrifugation; it was then labelled *in vitro* with [α^{32}P]dCTP by 'nick translation' (ref. 19), and sheared by sonication to an average size, as determined by agarose gel electrophoresis, of 500 base pairs. The DNA was heat-denatured and used to probe an excess of unlabelled single-stranded whole cell DNA from the same plasmid-carrying strains bound to nitrocellulose filters. The hybridisation was performed at 37 °C for 18 h in 2×SSC/50% (w/v) formamide under paraffin oil[17]. The values shown are the percentages of reassociation (±s.d.) of ^{32}P-labelled plasmid DNA with unlabelled plasmid DNA. Data are of the mean of eight separate determinations, after deduction of the values from control experiments using labelled probe and DNA from the plasmid-free host strains. These control values were about 5% of those for hybridisations with DNA from cells carrying the homologous plasmid.

result, TOL probe hybridised with more pMG18 bands than R2 (data not shown). These findings were confirmed in reciprocal experiments with pMG18 and R2 probes, which hybridised with the same resolved bands of TOL DNA, plus, in the case of pMG18, several additional bands (Fig. 1*b*). Since these represented small fragments, they were insufficient to account for the difference between 20 and 11 kilobases. However, some of the TOL bands which showed homology with both R plasmid DNAs were composed of more than one fragment, and comparison of the autoradiographs suggested that pMG18 probe was hybridising to a greater degree to some of those bands than did R2 probe.

The results of the NAH hybridisation against TOL DNA essentially confirm those of Heinaru *et al.*[10] for the SAL plasmid, which is very closely related to NAH: that is, homology was largely confirmed to a few bands (Fig. 1*b*). Of these, band 4 in the TOL fragmentation pattern, composed of one fragment species[21], is lost from the Tol$^-$ excision derivative of TOL, pWWO-8 (ref. 22). Only one TOL band showed some homology with all the probes. However, since this band was made up of more than one fragment species, we cannot say if the same fragment hybridised in each case: indeed, the homology could also be in different portions of one fragment.

When each of the R plasmid DNAs was used to probe the NAH digestion pattern, they only hybridised (relatively weakly) with the 5.8-kilobase band. This band was also one of those which hybridised with TOL probe. These findings suggest the existence of a very small 'core sequence' common to all four plasmids. Therefore, for these four plasmids of the IncP-9 group, incompatibility cannot be taken to imply more than a very limited amount of evolutionary relationship.

We thank Clive Duggleby for discussions, Jo Rennie for photography and the MRC for support.

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Received 20 April; accepted 20 June 1979.

1. Sagai, H. *et al. Antimicrob. Agents Chemother.* **10,** 573–578 (1976).
2. Jacoby, G. A. in *Microbiology—1977* (ed. Schlessinger, D.) 119–126 (American Society for Microbiology, Washington, 1977).
3. Korfhagen, T. R., Sutton, L. & Jacoby, G. A. in *Microbiology—1978* (ed. Schlessinger, D.) 221–224 (American Society for Microbiology, Washington, 1978).
4. Hansen, J. B. & Olsen, R. H. *Nature* **274,** 715–717 (1978).
5. Grindley, N. D. F., Humphreys, G. O. & Anderson, E. S. *J. Bact.* **115,** 387–398 (1973).
6. Willshaw, G. A., Smith, H. R. & Anderson, E. S. *Molec. gen. Genet.* **159,** 111–116 (1978).
7. Kawakami, Y., Mikoshiba, F., Nagasaki, S., Matsumoto, H. & Tazaki, T. *J. Antibiot., Tokyo* **25,** 607–609 (1972).
8. Summers, A. O. & Jacoby, G. A. *Antimicrob. Agents Chemother.* **13,** 637–640 (1978).
9. Dunn, N. W. & Gunsalus, I. C. *J. Bact.* **114,** 974–979 (1973).
10. Heinaru, A. L., Duggleby, C. J. & Broda, P. *Molec. gen. Genet.* **160,** 347–351 (1978).
11. Williams, P. A. & Murray, K. *J. Bact.* **120,** 416–423 (1974).
12. Jacoby, G. A. *Antimicrob. Agents Chemother.* **6,** 239–252 (1974).
13. Pemberton, J. M. & Clark, A. J. *J. Bact.* **114,** 424–433 (1973).
14. Palchaudhuri, S. & Chakrabarty, A. *J. Bact.* **126,** 410–416 (1976).
15. Austen, R. A. & Dunn, N. W. *Aust. J. biol. Sci.* **30,** 357–366 (1977).
16. Jacoby, G. A. & Matthew, M. *Plasmid* **2,** 41–47 (1979).
17. Roussel, A. F. & Chabbert, Y. A. *J. gen. Microbiol.* **104,** 269–276 (1978).
18. Guerry, P., LeBlanc, D. J. & Falkow, S. *J. Bact.* **116,** 1064–1066 (1973).
19. Maniatis, T., Jeffrey, A. & Kleid, D. G. *Proc. natn. Acad. Sci. U.S.A.* **72,** 1184–1188 (1975).
20. Denhardt, D. T. *Biochem. Biophys. Res. Commun.* **23,** 641–646 (1966).
21. Duggleby, C. J., Bayley, S. A., Worsey, M. J., Williams, P. A. & Broda, P. *J. Bact.* **130,** 1274–1280 (1977).
22. Bayley, S. A. *et al. Molec. gen. Genet.* **154,** 203–204 (1977).

Printed in Great Britain by Macmillan Production Ltd., Basingstoke, Hampshire

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